GENETICAL STUDIES ON THE CULTURED TISSUES AND THE REGENERATES IN THE GENUS *NICOTIANA*

HISAKAZU OGURA

1977

GENETICAL STUDIES ON THE CULTURED TISSUES AND THE REGENERATES IN THE GENUS NICOTIANA

HISAKAZU OGURA

1977

Contents

Chapter 1. INTRODUCTION

Haberlandt (1902) stated clearly the desirability of culturing the isolated vegetative cells of higher plants: "To my knowledge, no systematically organized attempts to culture isolated vegetative cells from higher plants in simple nutrient solutions have been made. Yet the results of such culture experiments should give some intersting insight into the properties and potentialities which the cell as an elementary organism processes. Moreover, it would provide information about the inter-relationships and complementary influences to which cells within the multicellular whole organism are exposed." Since experiments along these lines had been started by Haberlandt in 1898, little progress was made during the 30 years following his work.

The actual indefinite culture of plant tissues was accomplished indefinitely by White (1939a) in stem segments of Nicotiana hybrid and Gautheret (1939) and Nobecourt (1939) in carrot roots. These successes were mainly due to the replacement of the mature and entirely differentiated cells which Haberlandt and his coworkers had worked, by tissues containing undifferentiated and embryonic cells. It was also important that natural auxin (indole-3-acetic acid) was already available at that time.

Thereafter, de Ropp (1955), Torrey (1957), Melchers and Bergmann (1959), Jones et al. (1960) and Bergmann (1960) made attempts to establish single cell suspension culture and to observe division of single cells. Bergmann (1960) first succeeded in plating plant cells using the calluses of tobacco and kidney-bean. Steward et al. (1958) successfully restored the adult carrot plant from single cells or small cell aggregates derived from carrot roots. Takebe et al. (1971) ascertained the totipotency of single cells to adult plant in Nicotiana tabacum in a more strict sense, since procedures of protoplast isolation were established at that time. After the discovery of kinetin (Miller et al. 1955), Skoog and coworkers have persistently carried out a series of investigations on chemical control of growth and organ redifferentiation of tobacco (N. tabacum cv. Wisconsin 38) callus by the combinations of auxins and cytokinins.

As to the cytogenetical problems of plant cultured tissues, there have been different types of results; one type put emphasis on the appeatance of the aneuploid cells (Straus 1954, de Torok and White 1960, Fox 1963, Shimada and Tabata 1967, Norstog et al. 1969, Heinz et al. 1969 and others). Whereas,

another type pointed out that the majority of callus cells are eudiploid or polyploid (Skoog 1954, Cooper et al. 1964, Torrey 1967, Butcher et al. 1975 and others). Regarding to the chromosomal constitution of regenerated plants from callus cultures, the results of cytological observations are also classified into two different types; many investigators reported the eudiploid or polyploid constitution of original regenerates (Mitra et al. 1960, Blakely and Steward 1964, Venketeswaran and Spiess 1964, Singh et al. 1972, Novak and Vyskot 1975). Whereas, several studies stressed the aneuploid or mixoploid consitution of the regenerates (Sacristan and Melchers 1969, Niizeki 1974, Ogura 1975a, 1976).

In the present dissertation presented are the results of some continuous steps of plant tissue culture, namely, callus induction, shoot redifferentiation, analysis of the original regenerates and their offspring etc., in viewpoint of cytogenetics, using Nicotiana tabacum throughout as the material. In the beginning part of this thesis dealt with is the establishment of a redifferentiation system by application of a morphactin, chlorflurenol-methylester (CFM), combined with kinetin. In the later parts reported are cytogenetic analysis of regenerated plants and their offsprings,

and the relationships between the differences in genetic background and callus formation or shoot redifferentiation. An attempt to combine plant tissue culture with cytogenetics will be the implied subject matter of the present dissertation.

Chapter 2. LITERATURE REVIEW

The conception of plant tissue culture can be traced back to Haberlandt (1902), when he first proposed the idea of cell culture in isolated photosynthetic cells from the foliage leaves of Eichhorina crassipes. He was not entirely successful, but foresaw the use of cell culture as a potential means of studying physiological and morphological problems. His unsuccessful attempts to culture isolated vegetative cells from higher plants stimulated later investigators to cultivate tissues, organs and single isolated cells. His failure was probably due to the fact that he attempted to culture mature cells and the nutrients used may not have been adequate, however, he originally predicted the totipotency to regenerate a whole plant, and this was ascertained in 1958 by Steward and his coworkers, and in a more strict sense, in 1971 by Takebe et al. Little progress was made during the 30 years following Haberlandt's work. In 1922, Kotte, a Haberlandt's student and Robbins reported the successful cultivation of excised roots of pea, corn etc. These workers were able to culture roots for several weeks but not for an indefinite period. They, at least, demonstrated the possibilities of growing meristematic tissues by adding

several simple compounds to the media. In 1934, White reported the successful cultivation of tomato root-tips on the artificial media containing sucrose, inorganic salts and yeast extract, for an indefinite period and in fact, the cultures have been maintained continuously from that time to the present. Also in 1934, Gautheret reported that pieces of cambium derived from Salix capraea, Populus nigra and other trees continued to proliferate for some months. White (1937) indicated the importance of thiamine for the growth of cultured roots and in the same year, Went and Thimann reported the importance of auxin in the control of plant growth. Robbins and Bartley (1937) reported a successful cultivation of excised tomato roots for a definite period, using thiamine as one of the component of the media. Bonner and Addicott (1937) were successful in culturing excised pea roots for several months. In 1939, White, Nobecourt and Gautheret, independently of one another, reported the first unlimited culture of callus tissue. Thus, for the first time, unlimited culture of carrot explants (Gautheret, Nobecourt) and procambial tissue cultures of young stem segments of the hybrid Nicotiana glauca x N. langsdorffii (White) was achieved. These successes were mainly due to the replacement of the mature and largely differentiated cells by tissues that contain

meristematic and embryonic cells. Nobecourt's pioneer studies on carrot had shown that tissue cultures could differentiate roots, whereas White (1939b) described the development of leafy buds when his tissue culture of N. glauca x N. langsdorffii was transferred to liquid medium. However, no immediate progress was made towards identifying the factors controlling such organogenesis.

Since 1939, many significant contributions have been made: Van Overbeek et al.(1941) first reported the crown gall production by bacteria-free tumor tissues. Since that initial discovery, coconut milk has benn widely used in plant tissue culture media. During the period from 1943-1950, various fundamental studies, especially on nutritional problems were made (White 1943, 1949, Hildebrandt et al. 1946; Caplin and Steward 1949). In 1948, Skoog and Tsui suggested that the growth and bud formation of tobacco stem segments and callus tissues were chemically controlled by the combinations of adenine and naphthalene-acetic acid (NAA). During a decade of 1951-1960, various important findings were accumulated in this field. In 1952, Morel and Martin first described the method of obtaining virus-free clone from virus-infected apical meristem of dahlia plants. Muir et al. (1954) isolated single cells from friable callus masses of crown-gall origin and

placed these singly on the upper surface of filter-paper squares contacted with the "nurse" callus of the same strain. Thereafter de Ropp (1955) and Torrey (1957) tried to observe division of single plant cells in culture using microchamber or cover-slip method. Working with a series of experiments on growth and organ formation in tobacco callus, Miller et al. (1955) reported a new type of cell division factor from deoxyribonucleic acid (DNA) and termed this "kinetin". This substance exerted various biological effects on plants and tissue cultures in which regenerative ability of callus tissues was most noteworthy. In 1957, Skoog and Miller demonstrated in tobacco callus cultures that de- and redifferentiation of tissues can be controlled to some extent by the combination of kinetin and indole-3-acetic acid. Steward et al. (1958a, b, c) were successfully restored the adult plant from single carrot root cells using a liquid medium supplemented with coconut water. Bergmann (1960) first succeeded in plating plant cells derived from tobacco and kidney-bean calluses. In the same year, Cocking reported the release of protoplasts from root-tip cells using a fungal cellulase suspended in 0.6M sucrose solution. In 1959, Melchers and Bergmann cultured tissue derived from a haploid shoot of Antirrhinum majus. The tissue retained its haploid state

during several subcultures but then increased its ploidy level. The culture of microsporocytes had been tried in some species (Taylor 1950, Tulecke 1957, 1959). Haploid tissues and suspension cultures are clearly of particular interest for those interested in studying mutations. Guha and Maheshwari (1964) reported the embryoid formation in anther culture of Datura innoxia. Their further studies (1966, 1967) revealed these embryoids are of pollen origin. Production of haploid tobacco plants by anther culture was reported by Bourgin and Nitsch (1967), Nakata and Tanaka (1968) and Tanaka and Nakata (1969). In 1968, Niizeki and Oono reported a successful production of haploid rice plants by anther culture.

As to the cytological and cytogenetical problems of plant tissue cultures, Skoog (1954) reported the polyploidization of chromosome number in callus tissues of Nicotiana tabacum cv. Wisconsin No. 38. In the same year, Straus also reported the appearance of aneuploid cells in cultured tissue of maize endosperm. Thereafter a number of studies have been elaborated, suggesting there are some alterations or modifications of chromosome constitution in various cultured tissues, i.e., polyploidy (including haploidy), aneuploidy and chromosomal aberrations such as anaphase bridges, univalent laggards and fragments (Tulecke 1957, Torrey 1959, Mitra et al. 1960,

de Torok and White 1960). Fox (1963) reported in Nicotiana tabacum that three kinds of calluses of different autotrophy were screened and they showed a wide range of chromosome numbers varying from about 130 to 220 with more than 85% above the tetraploid level. Cooper et al. (1964) analyzed chromosome numbers in two 8-year-old clones of tobacco callus, both of which originated from single cells of a crown-gall callus. Countable mitoses revealed in both clones, numbers of 48 (2n), 96 (4n) and 192 (8n), indicating that both clones originated from cells having the normal diploid number of chromosomes. Aneuploidy in the callus cells of tobacco pith origin has also been stressed by Shimada and Tabata (1967). Tabata et al. (1968) examined the chromosome number of regenerated tobacco plants. Three regenerates obtained showed 48, 96 and 97 chromosome numbers, respectively. Only one regenerate normally formed 24 bivalent chromosomes at first metaphase of meiosis and showed normal characteristics. They considered this plant a normal diploid. Sacristan and Melchers (1969) also reported the aneuploid chromosome constitution of regenerated tobacco plants (morphologically abnormal and sterile) from a number of long-established aneuploid tobacco calluses. However, the majority of somatic chromosome number ranged within 60 to 80. Recently Ogura (1975a, 1976) and Niizeki

and Kita (1975) stressed the chimerism of somatic chromosome number in tobacco regenerates.

As cited before, the release of protoplasts by an enzymatic method was reported by Cocking (1960). Although Giaja in 1919 isolated protoplasts from yeast cells from the snail Helix pomatia, enzymatic isolation of protoplasts in higher plants became possible after 1960. During the decade of 1961-1970, several basic studies on plant protoplasts have been made (Ruesink and Thimann 1965, 1966, Cocking 1966, Takebe et al. 1968, Otsuki and Takebe 1969). However, protoplasts generally have not survived in culture on a defined medium, and then an important technical advancement was made by Nagata and Takebe (1970, 1971), who were able to obtain large quantity of viable protoplasts from tobacco leaves. Moreover, Takebe et al. (1971) have shown regeneration of whole plants from isolated mesophyll protoplasts in Nicotiana tabacum cv. Xanthi nc. Because they restored normal whole plants from single protoplasts through callus stage, this work is interpreted that in a very strict sense, adult plant regeneration from a single cell, irrespective of the origin of plant part, which Haberlandt had ever dreamed, becomes fully possible. A phenomenon of protoplast fusion in higher plants was first reported by Power et al. (1970). Production of auxotrophic mutants following

mutagenization of haploid cells was reported by Carlson (1969, 1970) in the fern Todea barbata and in Nicotiana tabacum. By combining the achievements of classical tissue and cell culture with recent techniques of protoplast isolation and fusion, and with the introduction of selective screening procedures, which were ordinarily used for microbes, Carlson et al. in 1972 have first produced somatic cell (or parasexual) hybrid between two different species of Nicotiana, N. glauca and N. langsdorffii. Smith et al. (1976) confirmed their results. Recently, Kao et al. (1974), Kao and Michayluk (1974) and Gamborg et al. (1974) have reported a method for interspecific and intergeneric fusion of protoplasts from several higher plant species and have discussed the possibilities of producing the somatic cell hybrids, which can never be obtained by sexual means. Several recent reports have discussed the actualization procedures of the induction and isolation of morphological or developmental mutants by combining tissue, cell and protoplast culture with mutagenesis in haploid higher plants (Dulieu 1972, Carlson 1973, Melchers 1974 and others). Moreover, some ideas concerning the trasformation of higher plants have presented, mainly based on the results of exogenous DNA uptake by plant protoplasts (Ledoux and Huart 1971, Ohyama et al. 1972, Doy et al. 1973).

As previously stated, one of the most unique features of several plant cells and tissues is their potential to redifferentiate into whole plants exhibiting the same phenotype as the original explants or cells. One of the most important contributions to this aspect is undoubtedly a group of works carried out by Skoog and his coworkers. After the discovery of kinetin, they clearly demonstrated that organ formation in tobacco tissue cultures can be controlled by varying the levels of IAA and kinetin in the nutrient medium. Skoog and Miller (1957) stated that a low kinetin/IAA ratio determines root formation whereas a high ratio results in shoot formation. Recently, Skoog (1970) reviewed his works with tobacco tissue cultures, indicating the role of kinetin and its relatives for redifferentiation. In searching effective factors controlling organogenesis, Kochhar et al. (1970, 1971) have observed that several tobacco smoke carcinogens exerted the morphogenetic effect like the combinations of IAA and kinetin on the stem-derived calluses of haploid tobacco plants. However, it proved ineffective on the callus from the diploid cultivar "Wisconsin No. 38". Ogura (1975a) has reported that a morphactin, chlorflurenol-methylester is effective in inducing organogenesis in pith-derived calluses of N. tabacum cv. Wisconsin No. 38. His

further studies (Ogura 1975b, c) revealed that some combinations of kinetin and chlorflurenol-methylester in an appropriate concentration resulted in 100% shoot redifferentiation in inoculated calluses. Although, demonstrations of such chemical control of organ redifferentiation in other undifferentiated tissues are still lacking.

The interrelationship of the diversity of manipulative techniques of plant tissue culture, which can be combined in a broad research program is diagrammatically summarized in Fig. 1. Even if we leave aside the new fields of mutagenesis in haploid cells and isolation of mutants, and somatic hybridization and transformation via protoplasts, all the steps shown in Fig. 1 seem to be still very unsatisfactory. Only in Nicotiana tabacum, all the steps represented in Fig. 1 appear partly to have come under man's control. One of the most important and promising steps is, undoubtedly, the establishment of artificially controlled systems which permit 100% redifferentiation. These systems will provide a powerful means to approach to many problems in plant physiology, biochemistry, genetics and breeding.

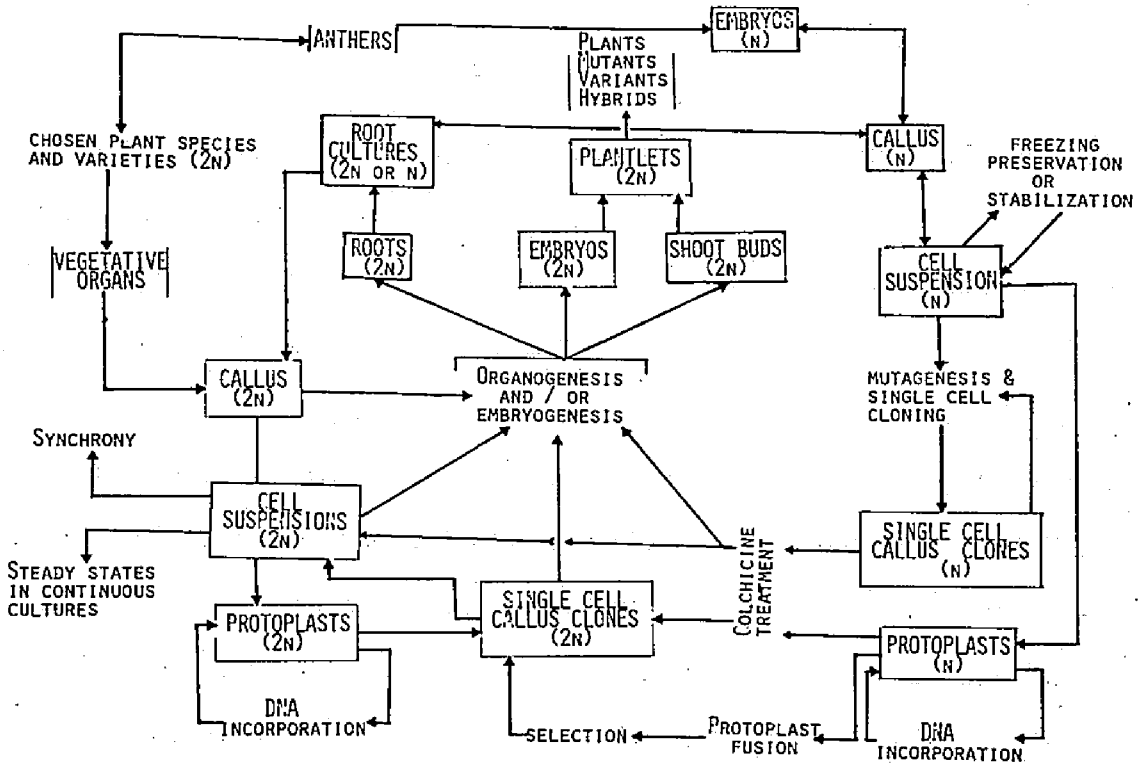


Fig. 1. Diagrammatic representation of the interrelationship among the techniques and methods of plant tissue and cell culture (modified from Street 1973).

Chapter 3. ESTABLISHING THE CONDITION FOR SHOOT REDIFFERENTIATION

Introduction

There have been many reports on the effects of auxins on dedifferentiation (callus induction) from various tissues and organs, and on the effects of cytokinins on redifferentiation (organ formation) from calluses in many kinds of species of higher plants. Auxins are known as one of the most effective exogenous factors for the induction of dedifferentiation (Skoog and Miller 1957, cf. Gautheret 1955). Cytokinins, in contrast, are generally recognized for their marked effect on the ability to regenerate shoots and/or roots in combination with, or without auxins from various kinds of calluses (Skoog and Miller 1957, Vasil and Hildebrandt 1967). Skoog and Miller (1957) demonstrated in a tobacco cultivar Wisconsin No. 38 that differnetiation of tissues can be controlled to some extent by the combination of kinetin (KIN) and indole-3-acetic acid (IAA). However, there remain many problems in establishing an artificially controlled system of redifferentiation, i.e., the system that can allow 100% shoot organogenesis.

One of the morphactins, chlorflurenol-methylester (CFM),

methyl-2-chloro-9-hydroxyfluorene-(9)-carboxylate, when combined with KIN, was found to be more effective than the combination of KIN and IAA, that was described by Skoog and Miller (1957), in shoot organogenesis from tobacco callus. Several combinations of KIN and CFM were successful for 100% shoot redifferentiation in inoculated calluses. In this chapter presented are synergistic interaction data between CFM and KIN on growth and shoot formation in tobacco callus.

Materials and Methods

To initiate and maintain the tobacco callus (Nicotiana tabacum cv. Wisconsin No. 38), the following was used: RM-1964 basal medium with 0.2 mg/l of KIN and 3.0 mg/l of IAA added (Skoog and Miller 1957). To test the morphogenetic effects of the combinations of CFM and KIN, pieces of stock calluses (ca. 500 mg each) were transferred, after four subculture passages to test tubes containing 10 ml of an RM-1964 basal medium with these growth regulators in various combinations. Ten callus pieces were tested for each combination. The composition of this RM-1964 medium is presented in Table 1. This RM-1964 basal medium consisted of RM-1964 inorganic salts (Linsmaier and Skoog 1965) supplemented with 0.4 mg/l of thiamine-HCl, 100 mg/l of myo-inositol and 3% sucrose (w/v)

and solidified with 1.0% agar (w/v). The pH of the medium was adjusted to 5.8 ± 0.1 before autoclaving. The shoot regeneration experiments were carried out at 25 ± 1 °C in the dark throughout. Callus weight was measured after 50 days of culture. Scoring the shoot-forming rate was made at 23, 32, 44 and 50 days of culture.

Results

Growth of callus tissues

In inoculated callus tissues, neither the single addition of KIN nor CFM was very effective for callus growth, and an addition of 10 mg/l or more was inhibitory. In contrast, some combinations of CFM and KIN resulted in comparatively vigorous growth of the inoculated calluses. These data are presented in Table 2.

Shoot regeneration from tobacco callus

Comparison of shoot-forming rate among the five different combinations of growth regulators is shown in Table 3. A combination of 1.0 mg/l of CFM and 2.0 mg/l of KIN proved to be successful for 100% shoot redifferentiation in inoculated calluses. Interaction data between CFM and KIN on the shoot-forming rate and the average number of shoots formed

Table 1. Composition of RM-1964 basal medium (Linsmaier and Skoog 1965)

Macroelements		Microelements	
Salts	mg/liter	Salts	mg/liter
A. Mineral Salts			
NH_4NO_3	1650	H_3BO_3	6.2
KNO_3	1900	$MnSO_4$ $4H_2O$	22.3
$CaCl_2$ $2H_2O$	440	$ZnSO_4$ $4H_2O$	8.6
$MgSO_4$ $7H_2O$	370	KI	0.83
KH_2PO_4	170	$NaMoO_4$ $2H_2O$	0.25
Na_2EDTA	37.3	$CuSO_4$ $5H_2O$	0.025
$FeSO_4$ $7H_2O$	27.8	$CoCl_2$ $6H_2O$	0.025
B. Organic Constituents			
Sucrose	30000	Thiamine-HCl	0.40
Agar	10000	Myo-Inositol	100

Table 2. Effects of CFM and KIN on growth and shoot formation in tobacco calluses.

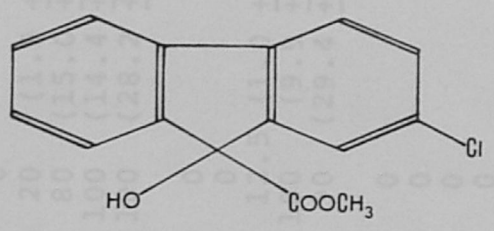
Growth regulator (mg/l)		Callus	Shoot
CFM	KIN	growth	formation
0	0	±	−
0	0.1	±	+
0	1.0	±	+
0	2.0	+	+
0	10.0	±	−
0.1	0	±	−
0.1	0.1	±	+
0.1	1.0	++	+
0.1	2.0	++	++
0.1	10.0	+	++
1.0	0	±	−
1.0	0.1	±	−
1.0	1.0	+	+
1.0	2.0	++	++
1.0	10.0	+	+
10.0	0	±	−
10.0	0.1	±	−
10.0	1.0	±	−
10.0	2.0	+	−
10.0	10.0	++	+
100.0	10.0	±	−

Callus growth: ±——++, weak to vigorous growth in various degrees.
Shoot formation: −, no formation; +, occasional formation;
++, consistent and vigorous formation.

Table 3. Comparison of shoot organogenecity among combinations of IAA and KIN, CFM and KIN and a single addition of KIN. Data were taken after 50 days of culture.

Growth regulator (mg/l)			No. calluses inoculated	No. calluses with shoots	Shoot-forming rate (%)
IAA	CFM	KIN			
1.0	0	2.0	15	2	13
0.1	0	2.0	12	11	92
0	1.0	2.0	20	20	100
0	0.1	2.0	10	10	100
0	0	2.0	10	8	80

in tobacco calluses after four different culture periods are presented in Table 4. The final observation was made 50 days after the initiation of the experiment. Results are shown in Fig. 2 and Tables 2 and 4. The highest rate (100%) of shoot redifferentiation was observed for the following combinations of CFM and KIN: CFM 0.1 mg/l + KIN 2 mg/l, CFM 0.1 mg/l + KIN 10 mg/l and CFM 1.0 mg/l + KIN 2 mg/l. The number of shoots formed was the highest for a combination of 1.0 mg/l of CFM and 10 mg/l of KIN. However, no significant difference was found between this combination and such other combinations as CFM 0.1 mg/l + KIN 10 mg/l or KIN 2.0 mg/l alone. An addition of CFM singly to the medium did not produce shoots, at least in 50 days of culture. However, formation of shoot-like bodies (SLBs) was occasionally observed at 0.1 mg/l or 1.0 mg/l dose of CFM. These SLBs were most frequently recognized at 0.1 mg/l of CFM, combined with doses of KIN higher that 1.0 mg/l. It was ascertained that some SLBs sometimes developed as shoots in more prolonged culture. SLBs were considered to be the primordial masses consisting of very small young shoots and/or deformed or transformed calluses. An example of the SLBs and shoots are seen in Fig. 4. An addition of singly KIN to the basal medium was effective in increasing the shoot number as well as in enhancing shoot



$C_{15}H_{11}ClO_3$

Fig. 2. Structural formula of a morphactin, chlorflurenol-methylester (CFM), methyl-2-chloro-9-hydroxyfluorene-9-carboxylate.

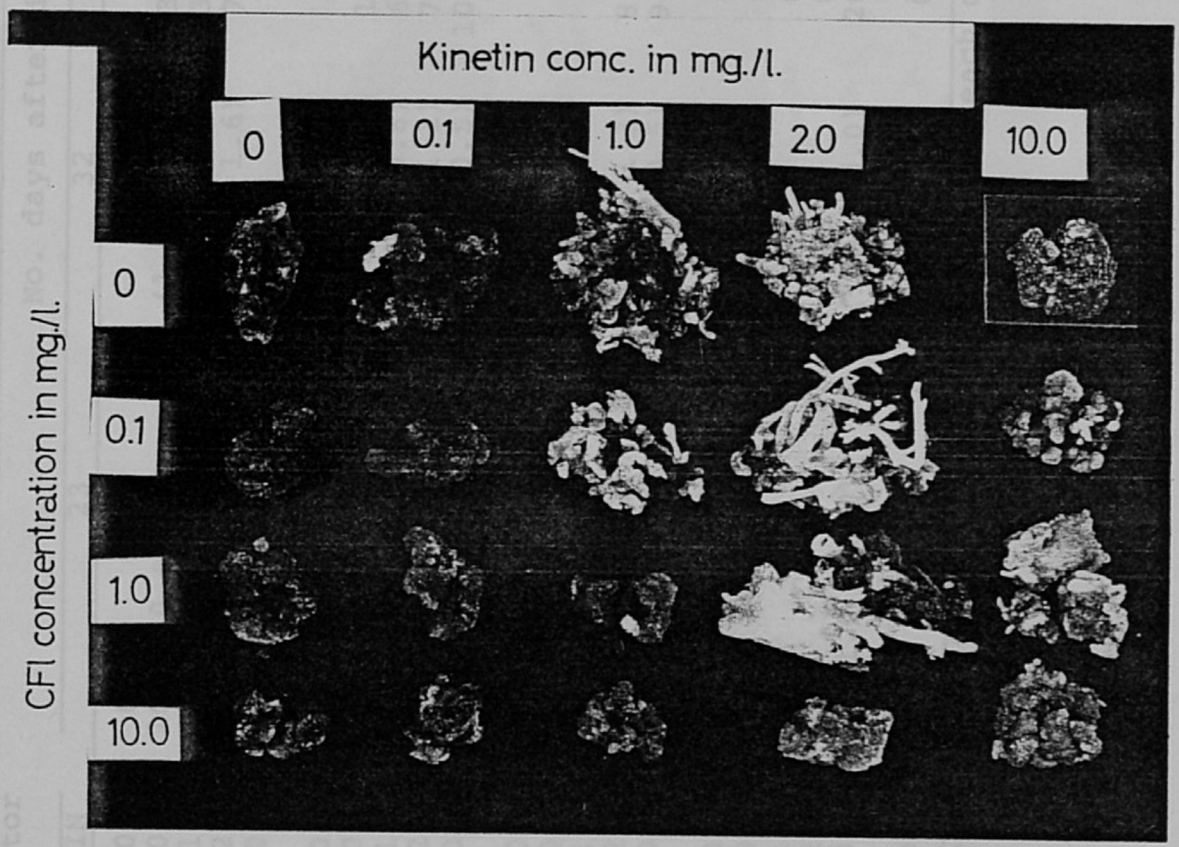


Fig. 3. Responses of calluses transferred to an RM-1964 medium with various combinations of CFM and KIN, after 50 days of culture.

Table 4. Percent of shoot-forming calluses and the average number of shoots formed per callus (in parentheses, ± S.E.) after four different culture periods.

Growth regulator (mg/liter)		No. days after inoculation			
CFM	KIN	23	32	44	50
0	0	0	0	0	0
0	0.1*	10 (1.0 ± 0.0)	12.5 (1.0 ± 0.0)	37.5 (2.0 ± 1.2)	42.9 (2.0 ± 0.6)
0	1.0	10 (1.0 ± 0.0)	20 (6.0 ± 0.0)	30 (22.0 ±14.0)	40 (21.3 ±12.3)
0	2.0	20 (1.5 ± 0.5)	60 (2.7 ± 1.6)	70 (19.7 ± 7.3)	80 (27.1 ± 7.8)
0	10.0	0	0	0	0
0.1	0	0	0	0	0
0.1	0.1	10 (1.0 ± 0.0)	10 (1.0 ± 0.0)	10 (1.0 ± 0.0)	20 (1.5 ± 0.5)
0.1	1.0	20 (1.0 ± 0.0)	50 (2.0 ± 0.8)	60 (14.3 ± 3.3)	80 (15.0 ± 4.6)
0.1	2.0	50 (2.0 ± 0.5)	50 (4.0 ± 1.1)	70 (13.6 ± 4.2)	100 (14.4 ± 3.6)
0.1	10.0	10 (1.0 ± 0.0)	60 (1.8 ± 0.5)	100 (14.3 ± 2.5)	100 (28.2 ± 5.6)
1.0	0	0	0	0	0
1.0	0.1*	0	0	0	0
1.0	1.0	0	0	0	12.5 (1.0 ± 0.0)
1.0	2.0	30 (1.3 ± 0.3)	50 (3.6 ± 1.3)	80 (4.1 ± 1.2)	100 (9.9 ± 3.6)
1.0	10.0	10 (1.0 ± 0.0)	50 (2.0 ± 0.5)	90 (16.9 ± 4.3)	90 (29.4 ± 6.4)
10.0	0	0	0	0	0
10.0	0.1	0	0	0	0
10.0	1.0	0	0	0	0
10.0	2.0	0	0	0	0
10.0	10.0	20 (1.0 ± 0.0)	20 (1.0 ± 0.0)	20 (1.0 ± 0.0)	20 (1.0 ± 0.0)
100.0	10.0	0	0	0	0

Ten callus pieces (ca. 500 mg each) were tested for each combination, except asterisked combinations (* nine callus pieces were tested).

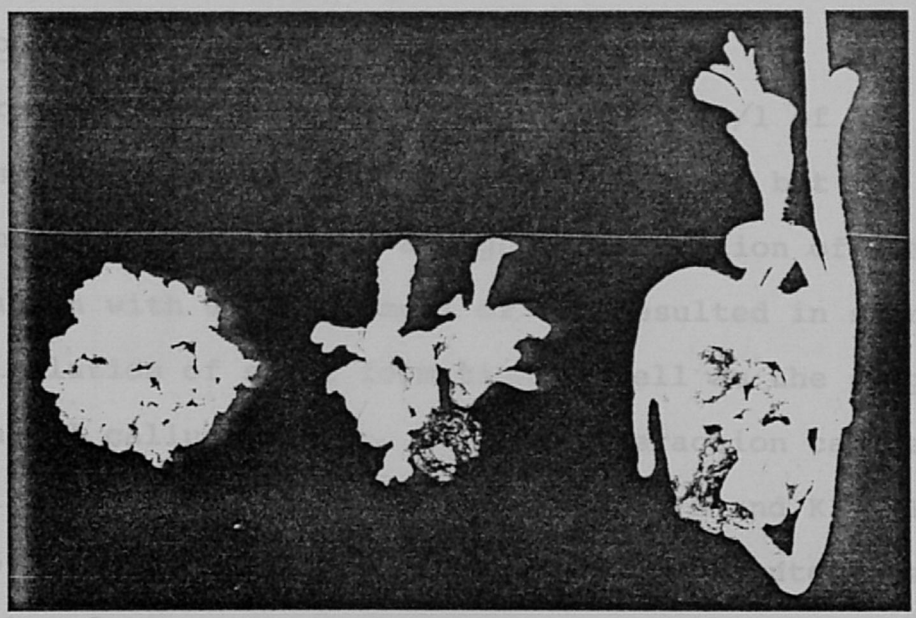


Fig. 4. Three different stages of morphogenesis in tobacco calluses at a combination of CFM (1.0 mg/l) and KIN (2.0 mg/l), after 50 days of culture. Shoot-like bodies are seen in the left callus (ca. x2).

formation; at least up to the dose of 2 mg/l. An addition of CFM and KIN in appropriate concentrations, however, was favorable for vigorous and consistent shoot formation.

Interaction of CFM and KIN in cultured tissues

As previously stated, an addition of 10 mg/l of KIN singly was inhibitory not only to callus growth, but to shoot formation; nevertheless a high concentration of KIN in combination with 0.1 or 1 mg/l of CFM resulted in significant stimulation of shoot formation as well as the slight enhancement of callus growth. Similar interaction can be detected in almost all the combinations of CFM and KIN (Tables 2, 4 and 5). These results indicate that CFM counteracts KIN in cultured tissues and that the appropriate balance between CFM and KIN leads to efficient shoot redifferentiation.

Morphology and histology of redifferentiating calluses

Growth of intact tobacco pith tissues explanted in a straight stock medium and in one added with 1.0 mg/l of CFM is shown in Fig. 5 and Table 6. These explants in both media began dedifferentiation within 10 days of incubation at 25°C in the dark. However, growth inhibition or retardation was clearly observed in the medium containing 1.0 mg/l of CFM, since the fresh weight increase in the tissues was more reduced in the stock medium, after 14 days of culture (Table 6).

Table 5. Effects of various CFM-KIN combinations in an RM-1964 medium on growth and shoot formation of tobacco calluses cultured in vitro. Data were taken after 50 days of culture.

CFM (mg/l)	Kinetin in mg/liter				
	0	0.1	1.0	2.0	10.0
0	No differentiation Slight growth	Occasional shoot formation	Shoots after 20-30 days	Shoots after 20-30 days	No differentiation Callus becomes hard
0.1	Occasional SLB formation	Occasioanal shoot formation	Shoots after 20-30 days SLB formation	Shoots after 20-30 days SLB formation	Occasional shoot formation SLB formation
1.0	Occasional SLB formation	No differentiation	Occasional shoot formation	Shoots after 20-30 days	Shoots after 20-30 days, Callus color light brown
10.0	No differentiation Little growth	No differentiation Little growth	Occasional SLB formation Little growth	No differentiation Callus color light brown	Occasional shoot and SLB formation, Callus color light brown

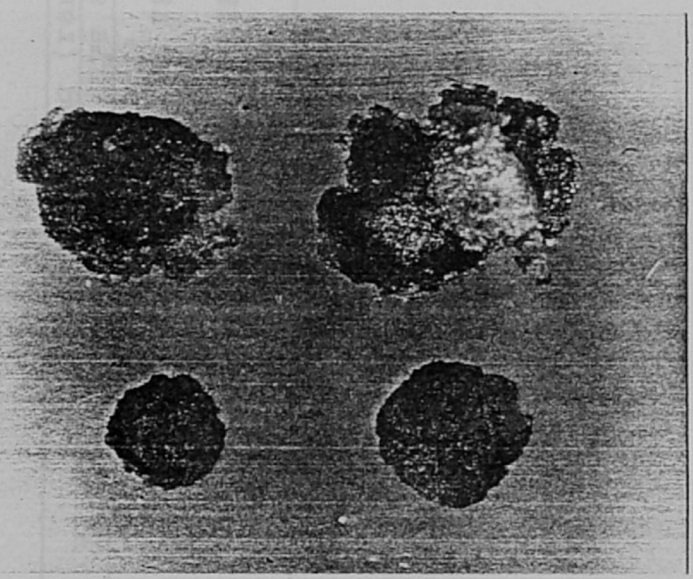


Fig. 5. Differences in the growth of intact tobacco pith discs inoculated in the ordinary stock medium (upper row) and a stock medium with 1.0 mg/l of CFM added (lower row), after 14 days of culture (ca. x2). Growth retardation by CFM is evident in the latter.

Table 6. Growth retarding effect of CFM on intact tobacco pith discs. Data are the averages of five discs.

Growth regulator (mg/l)			Fresh wt. in mg (range)	
IAA	KIN	CFM	Initial wt.	14 days after inoculation
3	0.2	1	150 (120-180)	785 (620- 930)
3	0.2	0	150 (120-180)	1280 (960-1550)

Morphological differences between an undifferentiated callus and shoot-forming calluses are seen in Fig. 6. Histological observation revealed that the development of tracheid-like bodies was much more pronounced in the latter than in the former ((Fig. 7).

Discussion

The present results clearly show that two groups of plant growth regulators, cytokinins and morphactins, regulate growth and shoot redifferentiation in tobacco callus. The data presented in Table 4 imply that when the ratio of KIN to CFM is 1 or larger, shoots are formed on the addition of CFM alone (0.1 or 1.0 mg/l). Although the regenerative ability of CFM has already been reported (Ogura 1975a), it is the balance between KIN and CFM that seems to be involved in shoot organogenesis. The stimulatory effect of CFM on shoot formation was promoted by KIN and vice versa. This interaction reminds us of the IAA-KIN interaction postulated by Skoog and Miller (1957). They concluded that a combination of IAA and KIN resulted in better growth and more vigorous shoot formation when compared with a single addition of either of them. In the present experiment, low levels of CFM with higher levels of KIN lead

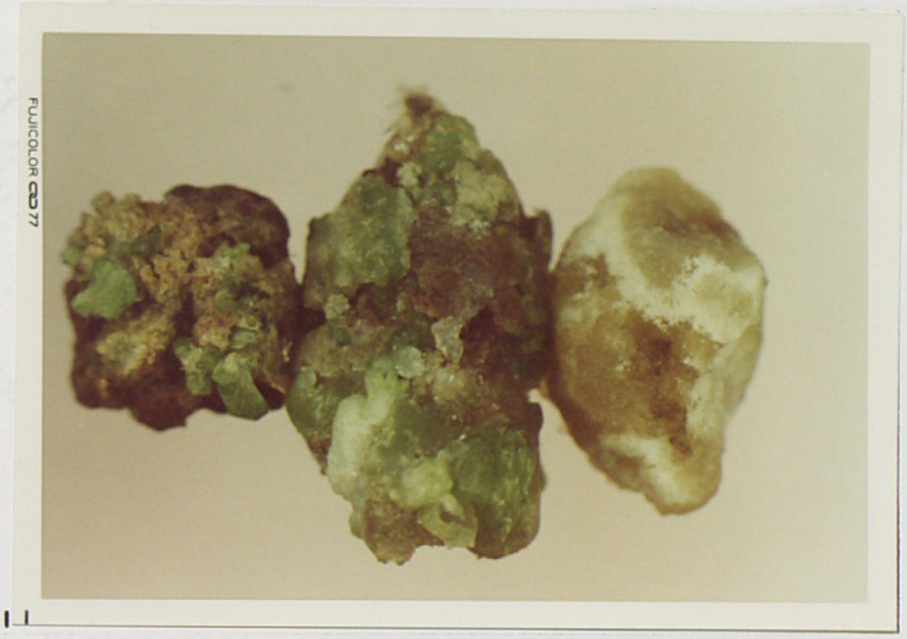
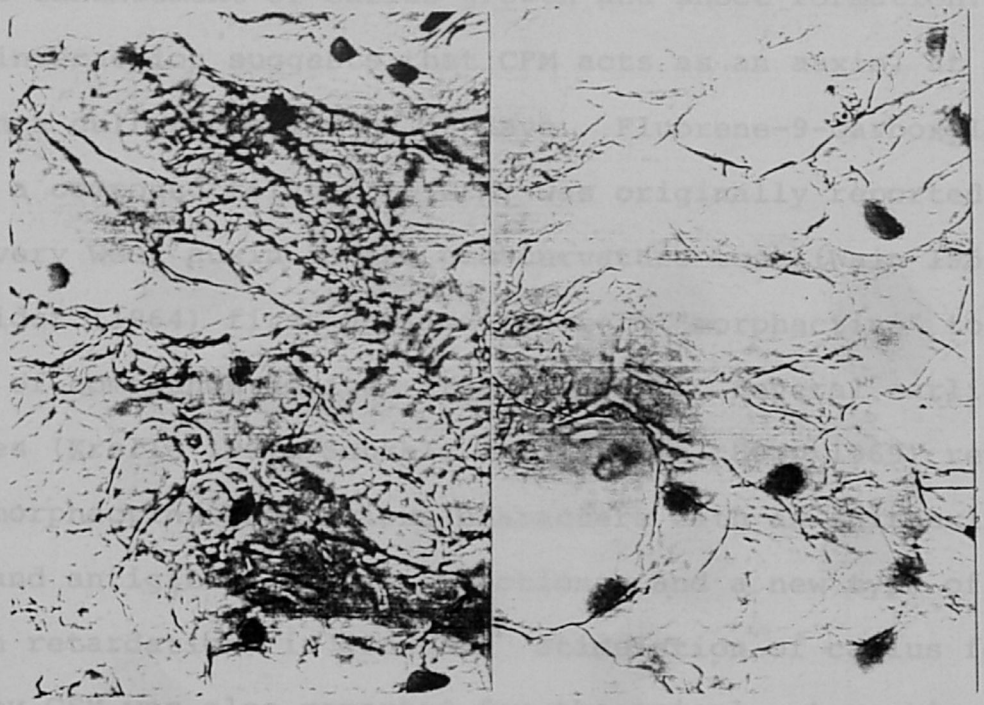


Fig. 6. Morphological differences between calluses inoculated in the shoot-regeneration medium (left and middle) in the ordinary subculture medium (right). Photographed about 30 days after inoculation in the shoot-regeneration medium (ca. x2.5).



(a) (b)

Fig. 7. Histological differences between calluses (x ca. 400) inoculated (a) in the shoot-regeneration medium (RM-1964 + CFM 1 mg/l + KIN 2 mg/l) and (b) in the sub-culture medium (RM-1964 + IAA 2 mg/l + KIN 0.2 mg/l). Note the ubiquitous development of tracheid-like bodies in (a).

to the enhancement of callus growth and shoot formation. This interaction suggests that CFM acts as an auxin, at least within a culture period of 50 days. Fluorene-9-carboxylic acid, a compound related to CFM, was originally reported to be a very weak auxin in the pea curvature test (Wain 1958). Schneider (1964) first applied the term""morphactins" to a group of compounds derived from fluorene. Several earlier studies (Krelle 1967, Sankhla and Sankhla, Bopp 1969) revealed that morphactins had various characters with an anitauxin-like and antigibberellin-like actions, and a new type of growth retardation, in general. Stimulation of callus formation by CFM was also reported for the apical cut sections of Phaseolus vulgaris, although the calluses formed by CFM differed from those induced by IAA or by gibberellin A_3 (Schneider 1967). Recently Schneider (1970) reported that the action of CFM and morphactins appear to be systematic and "polyvalent", and that they differ from the action patterns of other plant growth regulators. The present results are neither in accordance nor inconsistent with their findings. So far as the counter-action of CFM with KIN and the balance between CFM and KIN are concerned, however, the results can best be explained by assuming that CFM acts as an auxin in cultured tissues.

The concentration of CFM and KIN which had interacting

effects on shoot formation, seems to be higher than that of the IAA-KIN interaction described by Skoog and Miller (1957). Furthermore, no root formation was caused by any combination of CFM and KIN, at least during a 50 day culture period. Yet, SLBs were observed in several combinations of KIN and CFM. Some of these SLBs developed into shoots. The development of tracheid-like bodies in the callus was much more conspicuous in the "shoot-forming" medium as compared with an ordinary stock medium. It is assumed that these tracheid-like bodies have some relationship with shoot redifferentiation, although tracheid-like bodies are generallly recognized in undifferentiated calluses. Pieniazek and Saniewski (1968) reported that in twigs of Malus sylvestris CFM in combination with benzyladenine caused a marked increase in cambium activity and the promotion of phloem formation.

It has generally recognized that KIN and its related compounds manifest a marked effect in combination with, or without auxins on the regeneration of shoots and/or roots from various kinds of calluses (cf. Skoog 1970). However, the merit of using CFM, instead of auxins, as the counterpart of KIN consists in the higher and more consistent rate of shoot organogenesi. This seems to be the dual characters of CFM, acting as both an auxin and a cytokinin-like substance.

Summary

Pith-derived calluses of Nicotiana tabacum L. cv. Wisconsin No. 38 were inoculated on an RM-1964 medium containing various concentrations of a morphactin, chlorflurenol-methyl-ester (CFM) and kinetin (KIN). An addition of KIN (0.1 - 2 mg/l) alone was effective for shoot formation from the calluses, but high dose (10 mg/l) resulted in the inhibition of growth and in no differentiation. The inhibitory effect of a high dose of KIN was counteracted with CFM. Three combinations of KIN and CFM; CFM 0.1 mg/l + KIN 2.0 mg/l, CFM 0.1 mg/l + KIN 10 mg/l and CFM 1.0 mg/l + KIN 2.0 mg/l were successful for 100% shoot redifferentiation in inoculated calluses. An appropriate balance between CFM and KIN seems to be involved in shoot formation. The present results can best be interpreted by assuming that CFM acts as an auxin in cultured tissues.

Chapter 4. EFFECTS OF EXOGENEOUSLY SUPPLIED GROWTH REGULATORS ON CHROMOSOME CONSTITUTION

Introduction

Several cytological observations of tobacco regenerates from callus cultures have revealed that some of them exhibit chromosomally shimeric or mixoploid constitution (Sacristan and Melchers 1969, Collins et al. 1972, Niizeki and Kita 1975, Ogura 1975a, 1976). Whereas some investigators have made emphasis on the appearance of polyploid or aneuploid (not chimeric) plants (Murashige and Nakano 1967, Niizeki 1974, Novak and Vyskot 1975 and others). In this chapter presented is the relationship between media differences and the variation of chromosome number of regenerates.

Materials and Methods

Pieces of stock calluses (ca. 500 mg each) after four or five subculture passages were inoculated in test-tubes containing 10 to 15 ml of an RM-1964 basal medium supplemented with three groups of growth regulators (auxin, cytokinin and morphactin) in the following combinations and concentrations, in order to induce shoots:

(1) RM-1964 + 2 mg/l KIN (designated K plant)

(2) RM-1964 + 2 mg/l KIN + 0.1 mg/l IAA (KI plant)

(3) RM-1964 + 2 mg/l KIN + 0.1 mg/l CFM (KC plant)

In all cases, the media were adjusted to pH 5.8 ± 0.1 with NaOH solution, then sterilized by autoclaving for 20 min at 1.2 kg/cm^2 of atomospheric pressure. The shoot organogenesis experiment was carried out in the dark at 25 ± 1°C throughout. Regenerated vegetative shoots were then transferred to the RM-1964 basal medium containing 3 mg/l of IAA and 0.02 mg/l of KIN, to make them form roots under light. Some of the developing plantlets were transferred to pots filled with a mixture of sterilized vermiculite and soil. Some of them reached maturity and were self-pollinated by covering their flowers with paraffine paper bags to avoid cross-pollination. They were used for further investigation.

For cytological observation, root-tips of the redifferentiated plantlets and their offspring were pretreated with saturated 8-hydroxyquinoline solution (0.002M) for 2 to 2.5 hrs at ca. 15°C, then fixed with acetic alcohol (1 : 3). They were stained with 1% acetocarmine solution and squashed for microscopic observation.

Results and Discussion

Table 7 shows the chromosomal constitution of tobacco plants regenerated by the three combinations of KIN, CFM or IAA. Since almost all the regenerates from calluses, irrespective of the kinds of growth regulators in the medium, did show, more or less, chromosomal chimerism, each regenerate that manifested variable chromosome number of a limited range is regarded as "nearly stable" and is classified as follows:

	2n level	$2n=4x=48 \pm 12$ (36-60)
nearly stable	3n level	$2n=6x=72 \pm 12$ (60-84)
	4n level	$2n=8x=96 \pm 12$ (84-108)

chimeric — the regenerate that contained cells of other chromosome number which exceed each range

e.g., a regenerate which had cells of 36, 48, 60 chromosomes is classified into 2n level, however, a regenerate which had the cells of 48, 65, 75 chromosomes is classified into chimeric.

Percentage of chimeric regenerates was highest in the medium of single addition of KIN. IAA exerted some influences on reducing chimeric plants. Out of "nearly stabilized" plants, 3n level (2n=60-84) regenerates appeared most frequently. Sacristan and Melchers (1969) reported the aneuploid

Table 7. Comparison of shoot organogenecity and variation in chromosome number among the tobacco plants regenerated from the three different media.

Growth regulator (mg/l)			Shoot forming rate (%)	Chromosomal constitution of regenerates				Total	% chimerics
				Nearly stabilized			Chimeric		
IAA	CFM	KIN		2n level	3n level	4n level			
0	0	2	80	5	6	5	9	25	36
0.1	0	2	92	4	5	4	4	17	22
0	0.1	2	100	4	8	3	7	22	32

chromosome constitution of tobacco regenerates (morphologically abnormal and sterile) from a number of long-established aneuploid tobacco calluses. Countable mitoses revealed that the majority of somatic chromosome number ranged within 60 to 80. The present results are partially in accord with their results. The plants regenerated in the medium containing 0.1 mg/l of CFM and 2 mg/l of KIN sometimes exhibited abnormal characteristics, e.g., fused leaf (in their young stage of development), dwarfism, compact inflorescence etc. Abnormal compact inflorescences were sometimes observed in the medium containing KIN alone or a combination of KIN and IAA. However, fused leaf or syncotyl character was observed only when CFM was present in the medium. In fact, presoaked seeds of N. tabacum, N. sylvestris and N. tomentosiformis were treated with 1.0 mg/l of CFM solution for 72 hrs. Several forked leaf seedlings were obtained in N. sylvestris (Fig. 8). Cytological analysis of PMCs of CFM-treated N. sylvestris plants revealed that many of them were partiallly asynaptic (Fig. 9). As to this, Haccius (1969) reported a marked effect of CFM on the appearance of syncotyl seedlings in Eranthis hiemalis. The present results showed that CFM exerted a similar effect on tobacco regenerates, when CFM was applied to the cultured tissues, as well as to the presoaked seeds.

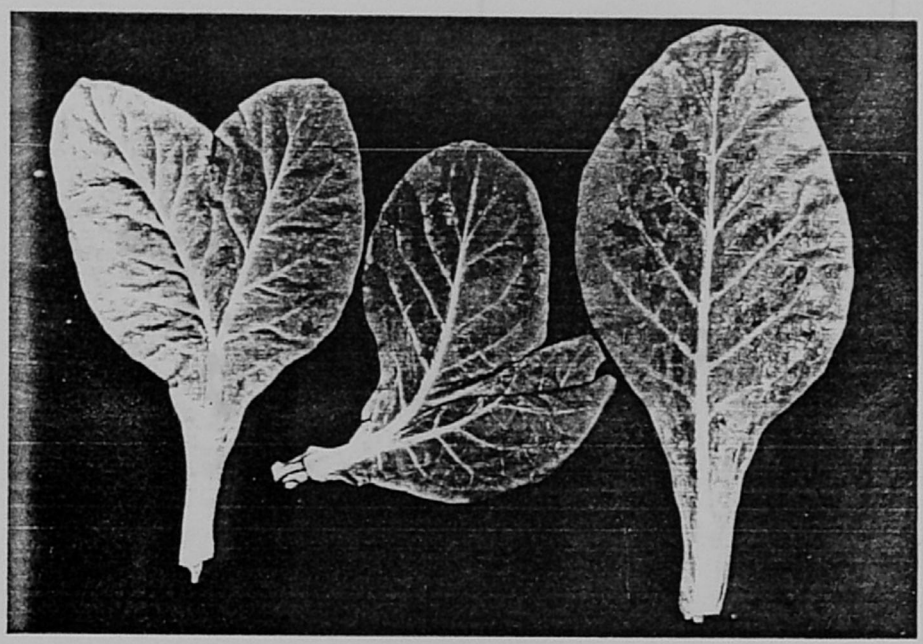
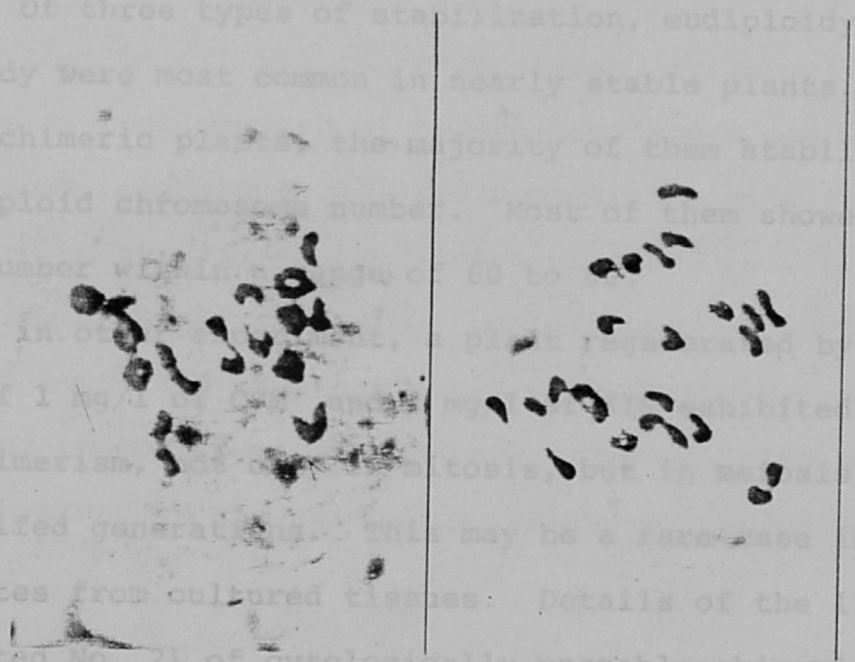


Fig. 8. Fused leaves (left and middle) and a normal leaf in CFM-treated N. sylvestris plants.



(a) (b)

Fig. 9. Chromosome configuration in metaphase I in PMCs in a CFM-treated N. sylvestris plants: (a) a PMC forming 12 bivalent chromosomes and (b) a PMC depicting partial asynapsis. Magnification ca. x 1100.

In the first selfed generation of chimeric as well as "nearly stabilized" plants, almost all of them stabilized at the normal (2n=48), polyploid or aneuploid chromosome number. Of three types of stabilization, eudiploidy and polyploidy were most common in nearly stable plants. In the case of chimeric plants, the majority of them stabilized at the aneuploid chromosome number. Most of them showed chromosome number within a range of 60 to 80.

However, in other experiment, a plant regenerated by a combination of 1 mg/l of CFM and 2 mg/l of KIN exhibited chromosomal chimerism, not only in mitosis, but in meiosis, through three selfed generations. This may be a rare case in the regenerates from cultured tissues. Details of the lineage (designated No. 2) of cytologically unstable chimeric strain through three generations are reported in later chapters. The results clearly showed that the regenerated tobacco plants exhibited a variation in chromosome number, irrespective of the kinds or combinations of growth regulators used for regeneration.

Summary

Chromosomal constitution of tobacco plants regenerated by an application of three different growth regulators in

appropriate combinations or in single application (KIN 2.0 mg/l, KIN 2.0 mg/l + IAA 0.1 mg/l and KIN 2.0 mg/l + CFM 0.1 mg/l) in the basal RM-1964 medium was cytologically studied. The observations of root-tip meristematic cells revealed that almost all the regenerates, irrespective of the kinds or combinations of growth regulators applied, were, more or less, chimeric. All the regenerates were further classified, according to the degree of chimerism, of at least five cells, into the following four groups; 2n level, 3n level, 4n level and truly chimeric or euchimeric. After selfing of the original regnerates, almost all the plants were stabilized at the normal, polyploid or aneuploid level.

Chapter 5. KARYOLOGICAL ANLYSIS OF ORIGINAL REGENERATES AND THEIR OFFSPRINGS.

Introduction

Cytological observations by a number of investigators have revealed that chromosome number of callus cells in several varieties of Nicotiana tabacum varies considerably. The results, however, are classified into two different types; one type has made emphasis on the appearance of the aneuploid cells (Fox 1963, Shimada and Tabata 1967), whereas the other type has pointed out that the majority of callus cells is polyploids ranging from 2n to 8n (Cooper et al. 1964, Murashige and Nakano 1967).

The variation of somatic chromosome number in tobacco regenerates has also beeen reported by Sacristan and Melchers (1969), Ogura (1975a, 1976) and Niizeki and Kita (1975). Tabata et al. (1968) and Zagorska et al. (1974) have reported their results on cytological analyses of PMCs at the first meiotic metaphse in the original regenerates. No imformations are yet available, however, on the chromosomal behavior of the offpsring of the regenerated plants. Data are here presented on the variation of chromosome number in root-tip meristematic cells and several other characteristics of original regenerates

and their selfed offspring.

Materials and Methods

Callus induction and shoot redifferentiation

For the initiation and maintenance of tobacco (N. tabacum cv. Wisconsin No. 38) callus , an RM-1964 basal medium (Linsmaier and Skoog 1965) added with 0. 2 mg/l of kinetin (KIN) and 3.0 mg/l of indole-3-acetic acid (IAA) was used (Skoog and Miller 1957). Pieces of the stock calluses (ca. 500 mg each) after three or four subculture passages were inoculated in test-tubes containing 10-15ml of RM-1964 basal medium supplemented with three groups of growth regulators (auxin, cytokinin and morphactin) in appropriate combinations and concentrations to induce shoots, as described later. The pH of the medium was adjusted to 5.8 ± 0.1 before autoclaving. The shoot regeneration experiment was carried out in the dark at 25 ± 1°C throughout, unless otherwise stated. Regenerated shoots were then transferred to the basal medium containing 3.0 mg/l of IAA and 0.02 mg/l of KIN to form roots (Skoog and Miller 1957) and placed under light. Some of the developing plantlets were transferred to pots filled with a mixture of sterilized

vermiculite and soil to grow in the greenhouse.

Regenerated tobacco plants

The regenerated plantlets were designated according to the kinds of growth regulators added to the basal medium from which they were induced: Nos. 1-6 and 10 represent the plants regenerated by a combination of 1.0 mg/l of a morphactin, methyl-2-chloro-9-hydroxyfluorene-(9)-carboxylate, chlorflurenol-methylester (abbreviated as CFM) and 2.0 mg/l of KIN (Ogura 1975b, c). IK1 shows a plant regenerated by a combination of 0.03 mg/l of IAA and 2.0 mg/l of KIN (Skoog and Miller 1957). K1 and K2 show those regenerated by addition of 2.0 mg/l KIN alone. S1 and S2 mean the first and second selfed offspring of original regenerates, respectively. To investigate the chromosome constitution of the offspring of the original regenerates, only Nos. 1 and 2 were used, their pedigree being shown in Fig. 10.

Cytology

Root-tips of the redifferentiated plantlets and their offspring were pretreated with chilled water (0-2^{o}C) for 24 hrs, and fixed with a mixture of ethanol and glacial acetic acid (3 : 1), or in some cases, with saturated (0.002M) 8-hydroxyquinoline solution for 2-2.5 hrs at 15^{o}C, then fixed. They were stained with 1% acetocarmine solution and squashed

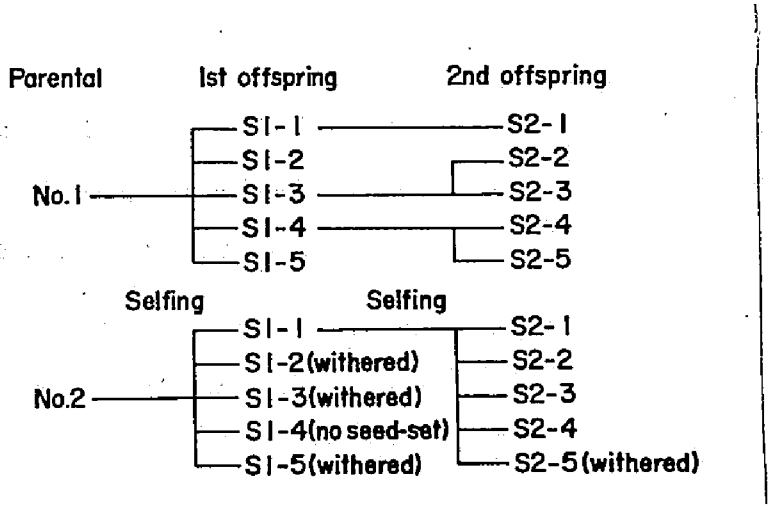


Fig. 10. Pedigree of original regenerates and their offspring.

for microscopic observation. The chromosome number was determined by sketching and photomicrographing all chromosomes. To observe anaphase cells, intact root-tips were directly fixed with acetic alcohol solution (3 : 1). For microsporocyte observation, the method described by Matsubayashi (1963) was used.

Characters observed

Several agronomic characters of regenerated tobacco plants and their offspring were observed; those include plant height, pollen and seed fertilities, number of days required for flowering. leaf color, leaf form, flower form and cytological features of meiosis. Plant height was measured when the flower buds became visible. Number of days required for flowering shows the period between transplanting from tissue culture media to pots and flowering of the plants in original regenerates, and that between the germination and flowering in the selfed offspring, respectively.

Results

Variation of chromosome number in original regenerates

Distribution of chromosome number in the root-tip meristematic cells of original regenerates is shown in Table 8. The data indicate that each regenerate shows a wide range of variation of its chromosome number, irrespective of the kinds or combinations of growth regulators supplemented for shoot regeneration. This suggests that regenerated tobacco plants are of cytologically chimeric or mixoploid nature, at least in the regions of the root-tip meristems.

Table 9 shows several characteristics of the original regenerates and their offspring. Out of all the original regenerates, only No. 1 appeared to be almost normal in most characteristics so far studied, although it showed a variation in its somatic chromosome number in the root-tip cells. The plant No. 1 exhibited a comparatively high pollen fertility (Fig. 11k) and mostly 24 bivalent chromosomes in the first diakinesis or metaphase of meiosis (Fig. 11f). The seed fertility of this plant was high, producing well-developed capsules containing numerous good seeds. In contrast, Nos. 2 to 6 and 10, K1, K2 and IK1 were shorter in height and had lower pollen fertilities than No. 1 and controls (seed-propagated normal plants

Table 8. Frequency of cells having various number of chromosomes in the root-tip cells of original regenerates.

Plant	Total no. cells examined	Mean ± S.E.	Range	Chromosome number									
				Less than 40	40-47	48	49-59	60-69	70-79	80-89	90-95	96	More than 96
No. 1	52	40.5 ± 1.7	16-84	21	13	14	2	1		1			
No. 2	50	43.6 ± 3.0	9-87	21	7	6	6	3	2	5			
No. 3	54	57.2 ± 2.8	15-96	12	7	4	7	6	11	4	2	1	
No. 4	51	57.8 ± 3.0	15-90	12	3	4	5	6	10	10	1		
No. 5	50	50.6 ± 2.9	19-96	15	4	11	3	6	2	7		1	
No. 6	53	58.3 ± 3.1	19-96	14	4	5	6	8	4	5	4	3	
No.10	53	69.2 ± 2.9	21-98	7	2	3	3	7	9	13	4	4	1
Subtotal	363	54.0 ± 1.2	9-98	102	40	47	32	37	38	45	11	9	1
K 1	52	68.4 ± 2.3	30-96	2	4	4	5	12	13	8	3	1	
K 2	50	66.0 ± 3.1	26-120	8	4	4	2	6	9	13	2	1	1
IK 1	51	53.6 ± 3.2	10-98	16	5	8	1	8	6	1	2	3	1

Table 9. Some characteristics of original regenerates and their offspring.

Plant	Chromosome number Mean ± S.E.	Chromosome number Range	Plant height (cm)	No. days for flowering	Pollen fertility (%)	Selfed seed fertility	Leaf color	Leaf form
No. 1	40.5 ± 1.7	16-84	97	86	79.0	subnormal	NG	N
No. 2	43.6 ± 3.0	9-87	56	168	33.6	low	DG	R
No. 3	57.2 ± 2.8	15-96	50	208	57.1	"	"	I
No. 4	57.8 ± 3.0	15-90	79	118	48.5	"	"	"
No. 5	50.6 ± 2.9	19-96	62	213	18.6	"	"	"
No. 6	58.3 ± 3.1	19-96	38	226	62.0	"	"	R
No. 10	69.2 ± 2.9	21-98	65	93	34.7	"	"	"
K 1	68.4 ± 2.3	30-96	--	--	--	--	"	I
K 2	66.0 ± 3.1	26-ca. 120	42	180	57.8	low	"	"
IK 1	53.6 ± 3.2	10-98	40	--	--	--	NG	"
No. 1S1-1			62	98	69.5	subnormal	NG	N
No. 1S1-2			89	106	69.2	"	"	"
No. 1S1-3	43.0 ± 1.0	19-92	91	97	91.7	"	"	"
No. 1S1-4			51	107	74.5	"	"	"
No. 1S1-5			42	99	81.6	"	"	"
No. 2S1-1	63.7 ± 1.9	16-92	55	102	47.2	low	DG	N
No. 2S1-4			28	280	0.0	none	"	I
No. 1S2-1			52	103	90.0	subnormal	NG	N
No. 1S2-2			51	95	79.9	"	"	"
No. 1S2-3	48.0 ± 0.0		39	107	89.3	"	"	"
No. 1S2-4			53	92	69.0	"	"	"
No. 1S2-5			55	98	75.2	"	"	"
No. 2S2-1	57.7 ± 1.9	36-72	52	140	48.8	low	NG	N
No. 2S2-2	53.6 ± 2.6	24-76	14	--	--	--	DG	R
No. 2S2-3	53.5 ± 2.1	24-73	67	164	48.1	--	"	N
No. 2S2-4	49.2 ± 1.8	34-68	31	--	--	--	NG	I

(Continued)

(No. 1 x W-38)F_1	-1		40	99	85.7	subnormal	NG	N
	-2	44.4 ± 1.0	24	111	82.9	"	"	"
	-3		54	95	89.6	"	"	"
(W-38 x No. 1)F_1	-1		38	98	72.7	subnormal	NG	N
	-2	48.0 ± 0.0	30	114	80.8	"	"	"
	-3		30	115	70.8	"	"	"
Contr. (W-38)	-1	48.0 ± 0.0	63	100	90.5	normal	NG	N
	-2	48.0 ± 0.0	60	102	88.5	"	"	"
	-3	48.0 ± 0.0	64	99	85.4	"	"	"

Note) NG; normal green, DG; dark green, N; normal, R; round, I; intermediate, W-38; normal Wisconsin No. 38.

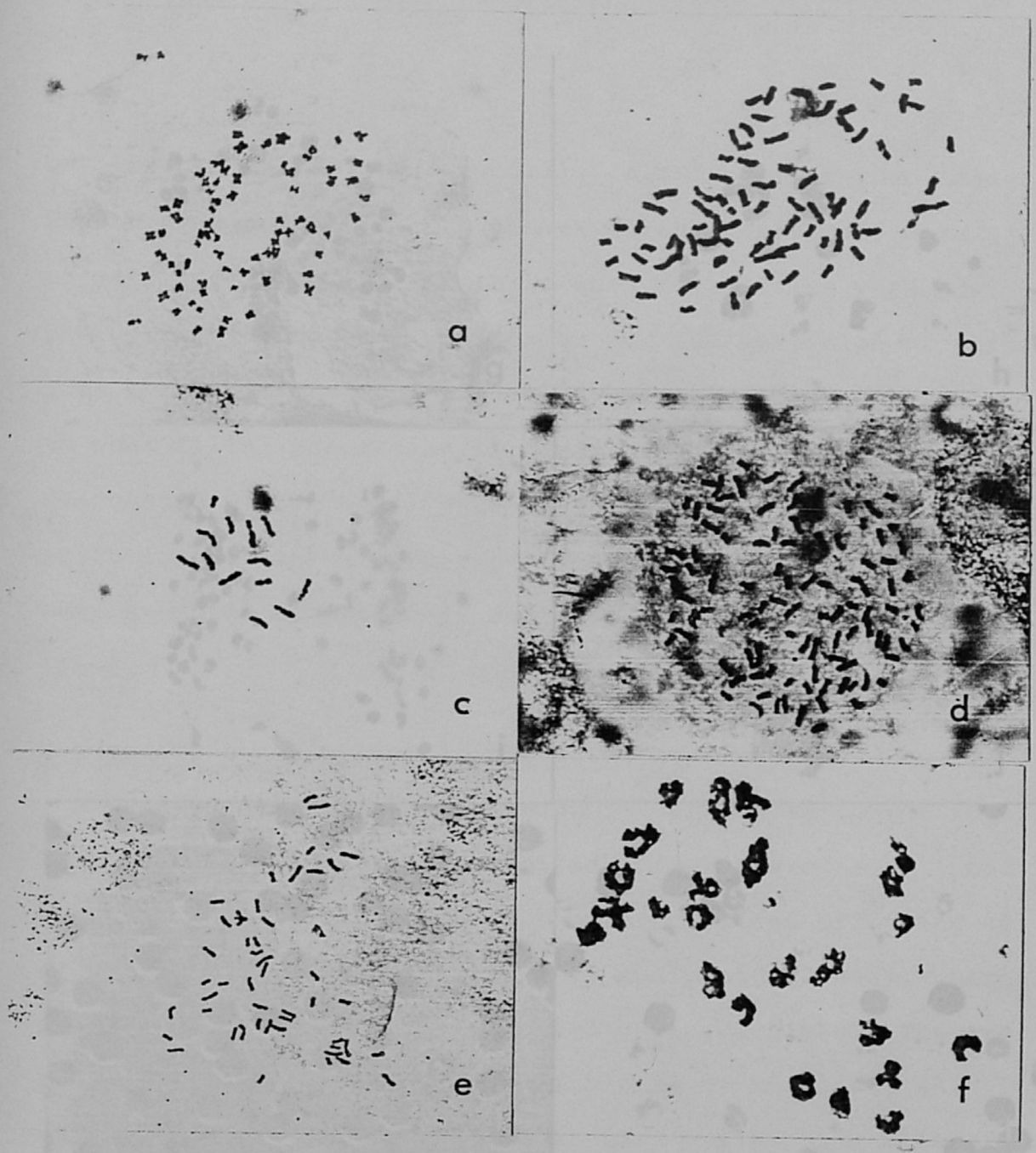


Fig. 11. (a-d) Metaphse plates of root-tip cells originated from the regenerated plants: (a) An aneuploid cell with 76 chromosomes (ca. x 1400), (b) 78 chromosomes (ca. x 2200), (c) 13 chromosomes (ca. x2300), (d) approximately 98 chromosomes (ca. x1500), (e) A metaphse plate of a root-tip cell from the No. 1 lineage, showing normal 48 chromosomes (ca. x800), (f) Diakinesis configuration of a PMC of the No. 1 lineage, showing 24 bivalent chromosomes (ca. x2000). (Continued to the next page)

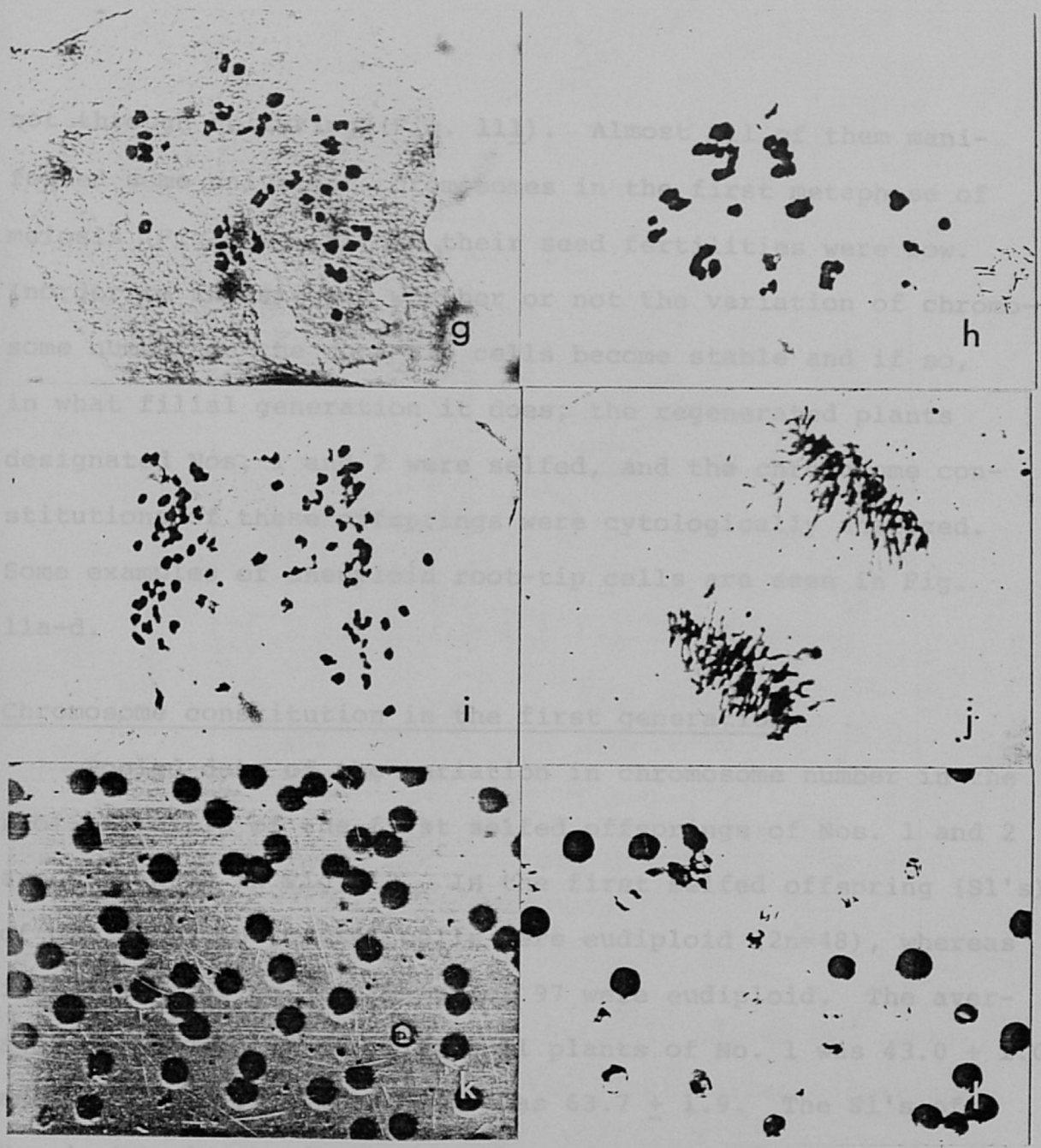


Fig. 11. (g, h) Metaphase configuration of PMCs in the No. 2 lineage, depicting some multi- or univalent chromosomes (g, ca. x1500; h, ca. x1800), (i) Meiotic anaphase of the No. 2 lineage, showing some anomalies (ca. x1400), (j) Anaphase bridges observed in mitosis in the No. 2 lineage (ca. x1400), (k) photomicrographs of pollen grains of the No. 1 lineage (ca. x240) and (l) of the No. 2 lineage (ca. x280).

not through culturing (Fig. 11i). Almost all of them manifested some univalent chromosomes in the first metaphase of meiosis (Fig. 11g, h) and their seed fertilities were low. Inorder to investigate whether or not the variation of chromosome number in the root-tip cells become stable and if so, in what filial generation it does, the regenerated plants designated Nos. 1 and 2 were selfed, and the chromosome constitutions of these offsprings were cytologically analyzed. Some examples of aneuploid root-tip cells are seen in Fig. 11a-d.

Chromosome constitution in the first generation

Pooled data of the variation in chromosome number in the root-tip calls of the first selfed offsprings of Nos. 1 and 2 are presented in Fig, 12. In the first selfed offspring (S1's) of No. 1, 50 out of 141 cells were eudiploid (2n=48), whereas in the S1's of No. 2, 19 out of 97 were eudiploid. The average chromosome number of five S1 plants of No. 1 was 43.0 $\pm$ 1.0 and that of two S1's of No. 2 was 63.7 $\pm$ 1.9. The S1's of No. 1 showed a tendency that the majority of cells had normal or hipodiploid chromosome numbers, in contrast with S1's of No. 2, in which the majority of the cells had hypodiploid numbers. Fig. 11e shows a cell with normal 48 chromosomes from a regenerated plants. Most characteristics of the

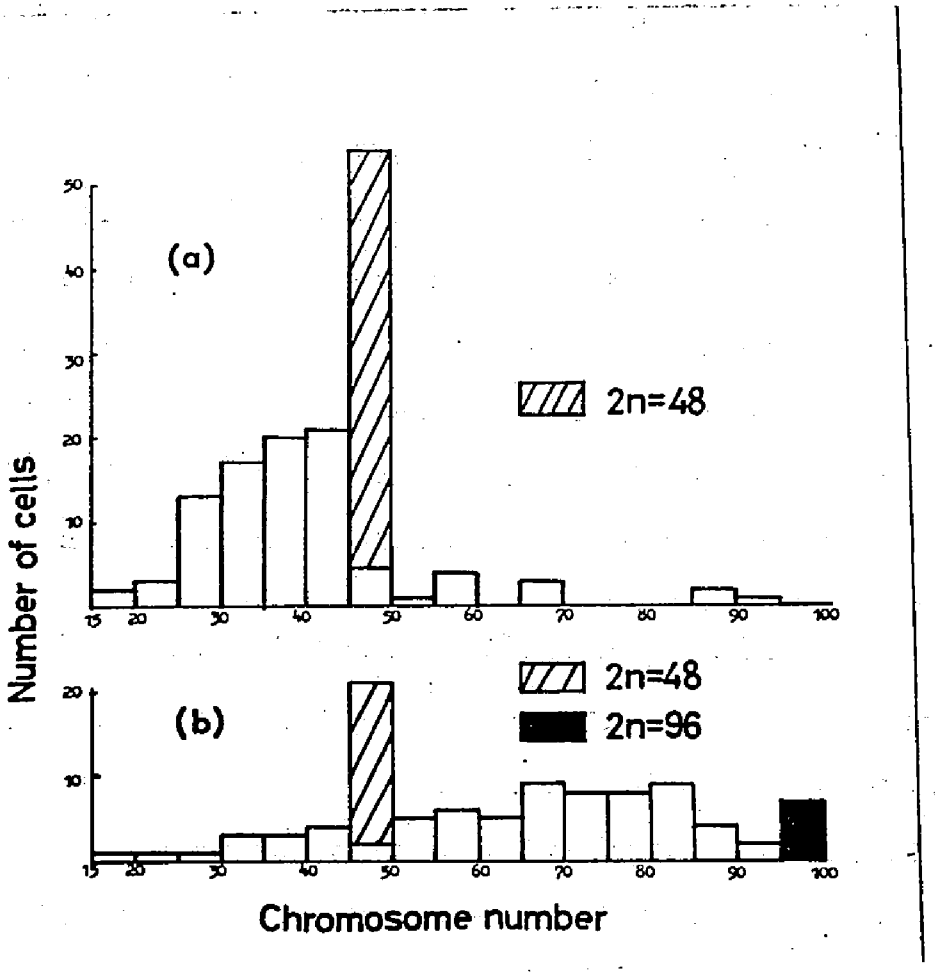


Fig. 12. Pooled data of the variation in chromosome number in the root-tip cells of the first selfed offpsring: (a) S1's of No. 1 and (b) S1's of No. 2. Total numbers of cells observed in S1's of Nos. 1 and 2 were 141 and 97 cells, respectively.

S1's of No. 1 were almost normal except for chromosome number (Table 9). In contrast, in the S1's of No. 2, three of five plants withered at young stage of development. Remaining two plants reached maturity and initiated flowering. However, a plant designated No. 2S1-4 was completely male-sterile and set no seeds (rf. Fig. 15). Another plant of No. 2(S1-1) produced some fertile pollen grains and its selfed capsules set some seeds. In general, the offspring of No. 2 was extremely unstable, and segregation of characters was frequently observed.

Chromosome constitution in the second generation

In the second selfed generation (S2) of No. 1, countable metaphase plates of root-tip mitosis revealed that almost all the cells had the normal chromosome number, as shown in Table 10. All the characteristics were almost normal as compared with those of the controls. In contrast, in S2's of No. 2, the chromosome number of all the offspring was still variable (Table 10). The data in this table indicate that the normal chromosome number (2n=48) is not the one most frequently found, and there is no clear modal chromosome number. Examples of aneuploid cells of the No. 2 lineage are shown in Fig. 11a-d. A plant No. 2S2-5 withered at young stage, and other characters

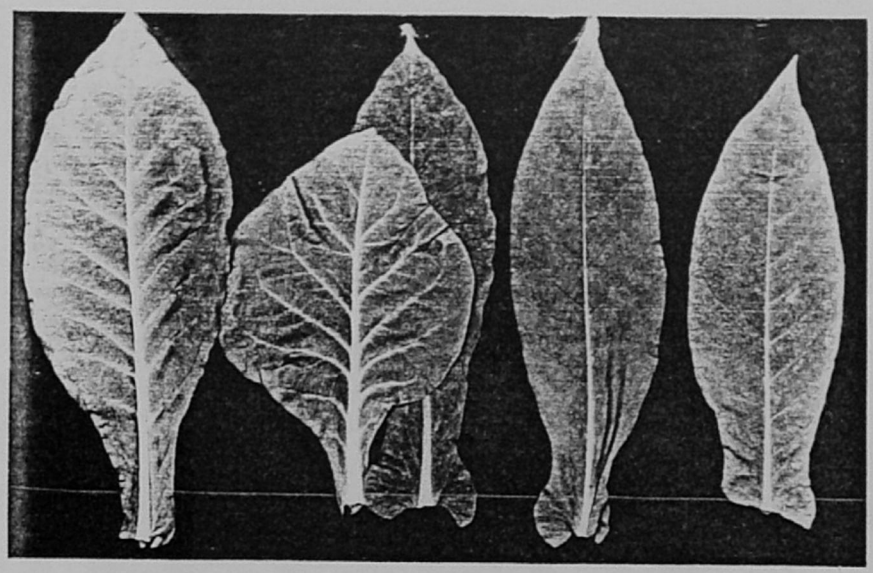
Table 10. Distribution of chromosome numbers in the root-tip cells in the second generation.

Desig-nation	Total no. cells examined	Mean ± S.E.	Range	Chromosome number					
				Less than 40	40-47	48	49-59	60-69	70-79
No. 1S2-1	17					17			
No. 1S2-2	20					20			
No. 1S2-3	20					20			
No. 1S2-4	15					15			
No. 1S2-5	20					20			
No. 2S2-1	30	57.7 ± 1.9	36-72	2	2	6	3	14	3
No. 2S2-2	30	53.6 ± 2.6	24-76	5	4	4	7	6	4
No. 2S2-3	32	53.5 ± 2.1	24-73	5	1	6	9	8	3
No. 2S2-4	31	49.2 ± 1.8	34-68	7	3	9	5	7	
Subtotal	123	53.5 ± 1.1		19	10	25	24	35	10

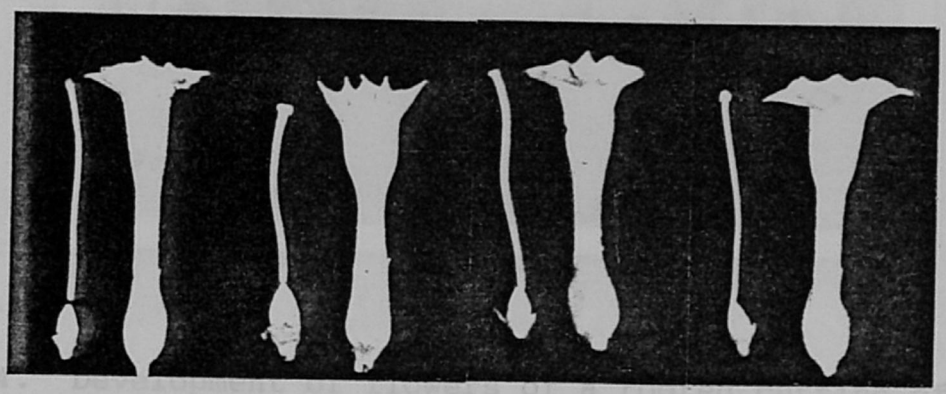
of S2's of No. 2 underwent a segregation. Their root-tip cells showed some anomalies in anaphase, such as bridges (Fig. 11j). Several lines of data presented indicate that there are some genetic and/or physiological factor(s) involved in abnormal cell division in the original regenerates and the offspring in the lineage of No. 2.

Other characteristics of the original regenerates and their offspring

As presented in Table 9, several characteristics, such as pollen and seed fertilities, and leaf color and form segregated in every generation of the No. 2 lineage. An example of round leaf is shown in Fig. 13a. Furthermore, original No. 2 had larger pistils and ovaries as compared with the controls or No. 1 (Figs. 13b and 14) and these traits were transmitted to the offspring, although this characteristics sometimes occurred in tobacco plants regenerated by a simple addition of KIN. A segregation of dwarf or growth-retarded plants was observed in the offpsring of No. 2 (Fig. 15a). Also, an abnormal compact inflorescence shape (Fig. 15b) sometimes occurred in the progeny plants of No. 2, especially in the dwarf plants. In contrast, in the lineage of No. 1, few abnormal characters as occurred in the No. 2 lineage were observed. However, the F_1s derived from the crosses between No. 1 x W-38 showed a



(a)



(b)

Fig. 13. (a) Typcial leaf form of regenerated and normal tobacco plants. Left to right; No. 1 , No. 2, Burk's F_5 (F_5 progeny of synthesized amphiploid between N. sylvestris and N. tomentosiformis, originally produced by Dr. L. G. Burk) and normal Wisconsin No. 38 tobacco. (b) Typical floral morphology of regenerated and normal plants. The arrangement of flowers is in the same order as in (a).

Fig. 14. Development of flowers of a rooted cutting of No. 2 (left) and normal Wisconsin No. 38 (right) (ca. x 1/2).

(a) (b)

Fig. 15. Young and mature tobacco plants of the first selfed offpsring in the No. 2 lineage. (a) Left; No. 2S1-4, right; No. 2S1-1. (b) Left; No. 2S1-1, tall, exceptional subnormal inflorescence: right; No. 2S1-4, dwarf, abnormal inflorescence resulted in no seed-set.

little variation of chromosome numbers, despite all the three F_1s of normal Wisconsin No. 38 x No. 1 exclusively gave normal chromosome number (2n=48), as presented in Table 9. Considering such difference as observed in reciprocal crosses and a fact that Sl's of No. 1 had some mixoploid numbers, the cytoplasmic effects on the stability of chromosome numbers may be taken into account.

Discussion

There have been numerous reports on the chromosomal chimerism in higher plants (Hollingshead 1932, Love 1938, Vaarama 1949, Watanabe 1962). Limiting to the studies on the genus Nicotiana, a great number of investigations have been made on cytological chimeras in cultured tissues (Fox 1963, Cooper et al. 1964, Shimada and Tabata 1967, Murashige and Nakano 1967) and in the regenerated plants (Sacristan and Melchers 1969, Ogura 1975a, 1976, Niizeki and Kita 1975), as well as in intact plants or hybrids (Moav 1957, Yang 1964, 1965, Gerstel and Burns 1966). However, only a few literature dealing with the progeny plants of regenerates derived from tissue cultures have been elaborated.

Sacristan and Melchers (1969) reported the aneuploid chromosome constitution of regenerated tobacco plants

in the first anaphase, aberrations like chromosome bridges were detected in some of the cells examined. Moreover, in the mitotic anaphase of No. 2 and its offspring, chromosome bridges were frequently observed (Fig. 11i). Such anomalies in the meiosis and mitosis will lead to an unequal distributions of chromosomes and to the formation of qualitatively different cells. As presented in Table 8, pollen and seed fertilities of No. 2 lineage were far lower than those of No.1 lineage and the controls. In addition, the author observed perishing of some offspring of No. 2 at their early stage of vegetative growth. All the survived progenies of No. 2 showed the variation in chromosome number and a continuous segregation of morphological characters in every generation so far studied. These observations indicte that the lineage of No. 2 is much more unstable both cytologically and physiologically, comparing to the lineage of No. 1 in which the variation in chromosome number stabilized to be normal after two cycles of selfing and the morphology was nearly normal.

Judging from the results that abnormal cell divisions were frequently observed in both meiosis and mitosis of the No. 2 lineage, whereas only a little cytological irregularities were observed in those of the No. 1 lineage, there may exist some gene(s) which disturbs both types of cell division.

(morphologically abnormal and sterile) from a number of long-established aneuploid tobacco calluses. Countable mitoses revealed that the majority of somatic chromosome number was within a range of 60 to 80. Working with haploid N. tabacum and N. otophora plants obtained by anther culture, Collins et al. (1972) reported that 14 out of 63 regenerates in N. tabacum cv. White Burley and five out of 64 in N. otophora showed mixoploid constitution of somatic chromosomes. Zagorska et al. (1974) observed a wide range of chromosome number variation in meiosis of regenerated plants of N. tabacum cv. Trapezond, suggesting that the variation was caused by a high degree of aneuploidy in the nondifferentiated tissues and by the damages in the chromosome apparatus of the cells which produced the regenerates.

The present results showed that the regenerated tobacco plants exhibited a variation in chromosome number, irrespective of the kinds or combinations of growth regulator used for regeneration. In the plants (Nos. 1 and 2) regenerated by a combination of a morphactin and a cytokinin, the variation of chromosome number was inherited successively at least for three generations. The second selfed offspring of No. 1 had stabilized the chromosomal constitution to the normal level, whereas that of No. 2 still exhibited the cytological chimeras.

The data presented in Table 8 imply that cytological variations paralelled with other morphological and/or physiological characteristics to a considerable extent. The studies of the No. 1 and No. 2 lineage indicated that their difference may be genetical. As shown in Table 8, a little difference was observed between the reciprocal F_1s of No. 1 and normal plants on their cytological stability. This suggests that some cytoplasmic effects may also be involved in the chromosomal variations. In general, the present results show a situation of a considerable mixoploidy of origianl regenerates and their offspring in N. tabacum cv. Wisconsin No. 38. The high variability of the chromosome number of both regenerates and theri offspring seem to be attributable to (a) the chromosomal or genetic alternations induced by tissue culture, (b) the genetic alternations induced by growth regulators (CFM and KIN) used for regeneration or (c) the phylogenetical characteristics of N. tabacum. It is interesting that the lineage of No. 2 still shows the variation in chromosome number as well as continuous segregation of several characteristics even after two selfing cycles. In fact, in the microsporocytes of No. 2 and its offspring, disturbances of the first metaphase configuration such as as appearance of misparing of chromosomes were frequently observed in PMCs. Also

Among various studies on chromosomal chimerism in intact higher plants (mainly hybrids), several studies suggested the existence of a gene controlling spindle abnormalities which lead to abnormal cell division in Ribes nigrum (Vaarama 1949), in a variety of common wheat (Watanabe 1962) and in diploid hybrids of Solanum tuberosum (Mok and Peloquin 1975a). In the case of colchicine-treated Ribes plants, Vaarama (1949) ascibed "chromosomal mosaics" to the altered genic control induced by a permanent effect of colchicine. Watanabe (1962) assumed a gene inducing spindle abnormalities, although he added that a detailed study on the processes in early stages before diakinesis is required to determine the existence of a gene with certainty. Mok and Peloquin (1975a, b) reported three mechanisms (spindle abnormalities and two types of premature cytokinesis) of 2n pollen (diplandroid formation in diploid potatoes, and based on the results of test crosses and allelism tests, the three mechanisms of diplandroid formation are controlled by simple recessive genes induced by mutation. For the present assumption of the gene(s) involved in abnormal mitotic and meiotic cell division leading to cytological chimeras, it is required to perform reciprocal crosses between the plants of the No. 2 lineage and normal (intact) plants as weel as test crosses of the F_1 hybrids with each of their parents, which is dealt with in Chapter 6. More extensive

study is also required, for testing the effects of morphactins and cytokinins on induction of chromosomal chimeras of regenerated plants.

Summary

A cytological analysis was made with root-tip meristematic cells of regenerated plants and their offspring originated from tissue cultures of Nicotiana tabacum cv. Wisconsin No. 38. Each original regenerate showed a wide range of variation of chromosome number, irrespective of combinations or concentrations of growth regulators used for regeneration. Two regenerates designated Nos. 1 and 2 were used for the analysis of further generations. In the first selfed generations (S1), all the progenies of both Nos. 1 and 2 exhibited variable chromosome numbers. Five plants derived from No. 1 had 43.0 $\pm$ 1.0 chromosomes on the average, while two plants from No. 2, 63.7 $\pm$ 1.9. The majority of S1 plants of No. 1 showed normal or hypodiploid chromosome numbers, whereas hyperdiploid numbers were common in S1 plants of No. 2. In the second selfed generation (S2), the chromosome number of all the progenies of No. 1 stabilized to 48, whereas all the S2 plants of No. 2

exhibited still highly variable chromosome numbers. Moreover, all the plants belonging to the No. 1 lineage were almost normal physiologically (pollen and seed fertilities, plant height, leaf color, leaf form, inflorescence shape etc.). In the No. 2 lineage, in contrast, these characteristics segregated frequently, and most of the plants exhibited some anomalies. Observations of mitotic anaphase revealed that there exist considerable irregularities such as anaphase bridges. These results indicate that the lineage of No. 2 possesses some genetical factor(s) leading to the cytological instability. From the reciprocal crosses between No. 1 and normal plants, existence of a cytoplasmic effect on the cytological stabilization was also presumed.

Chapter 6. GENETIC CONTROL OF CHROMOSOMAL CHIMERISM FOUND IN A REGENERATE FROM TOBACCO CALLUS

Introduction

There have been numerous reports on chromosomal chimerism in higher plants (Hollingshead 1932, Love 1938, Vaarama 1949, Watanabe 1962, Fukumoto 1962, Omara 1976 and others). Limiting citations to studies on the genus Nicotiana, a number of investigations have been made on cytological chimeras in cultured tissues (Fox 1963, Cooper et al. 1964, Niizeki 1974, Novak and Vyskot 1975 and others), and in regenerated plants (Sacristan and Melchers 1969, Collins et al. 1972, Niizeki and Kita 1975, Ogura 1975a, 1976), as well as in intact plants or hybrids (Kostoff 1930, Yang 1965, Gerstel and Burns 1966 and others). Ogura (1976) reported that a plant regenerated by a combination of a morphactin, chlorflurenol (CFM) and kinetin (KIN) exhibited variation in its chromosome number, even in the second selfed generation. Since the chromosomal chimerism was transmitted to progeny plants, the chimerism was considered to be genetic. Segregation data on chmeric plants in the F_2 and B_1 generations in crosses between a chimeric plant and a normal one are presented here, and the mode of inheritance of the chimeric character is considered. In addition, two foliar

characters, i.e., the bilateral asymmetry in a leaf (Sakai and Shimamoto 1965) and intraplant variability of leaf form, were measured in those generations.

Materials and Methods

Crossing experiment

To investigate the mode of inheritance of the chimerism, a progeny plant of No. 2 (No. 2S2-4) was reciprocally crossed with a normal plant (W-38). These F_1s were selfed or backcrossed with each of their parents. The procedures used are diagrammatically presented in Fig. 16.

Cytology

Root-tips of the plantlets were pretreated with chilled water (0-2°C) for 24 hrs, and fixed with acetic alcohol (3 : 1), or in some cases, treated with saturated 8-hydroxy-quinoline solution (0.002M) for 2.5 hrs at ca. 15°C prior to fixation. They were stained with 1% acetocarmine solution and squashed for microscopic observation. The chromosome number was determined by sketching and photomicrographing all chromosomes. To determine whether a plant was chimeric, the chromosome number of at least five metaphase plates was carefully counted for each plant. For microsporocyte observation, the method described by Matsubayashi (1963)

was used.

Leaf character measurements

As illustrated in Fig. 17, all leaves at young developmental stage of plant in the field, were measured for their entire width (W), width of the left (L) and right (R) halves of the blades, and vertical length of the blades. On the basis of these measurements, leaf instability was expressed in two ways: (1) asymmetry index (A), or absolute difference between the widths of two halves of a leaf divided by the maximal width of the leaf, and (2) the foliar ratio (FR), or the ratio of vertical length to the maximal width of the leaf. They are formulated as follows:

$$A = |L - R| / W$$

$$FR = VL / W$$

Results

Seed-set and germination

The capsules of No. 2 and its offspring usually varied more in size and were smaller than those of normal plant (W-38). The capsules of (W-38 x No. 2S2)F_1 were generally bigger than the reciprocals. Examples of capsules of W-38, selfed progenies

of No. 2 in the second generation (No. 2S2), their F_1s and B_1s are shown in Fig. 18. In the B_1 generation, the capsules of the B_1s backcrossed with W-38 were bigger than those backcrossed with No. 2S2. The degree of seed-set of the capsules closely paralleled with the size of the capsules. Among the four B_1 combinations, the degree of seed-set was of the following order (from high seed-set): (W-38 x No. 2S2)F_1 x W-38, (No. 2S2 x W-38)F_1 x W-38, (W-38 x No. 2S2)F_1 x No. 2S2 and (No. 2S2 x W-38)F_1 x No. 2S2. The germination rate of the latter two combinations was also very low, while that of the former two combinations was not so low. In addition, selfed progenies of No. 2 frequently produced capsules of variable shape. This suggests that the lineage of No. 2 possesses some genetic abnormalities involved in capsule size and seed germination.

Segregation of chimeric individuals in the F_2 and B_1 generations

A progeny plant of No. 2, No. 2S2-4 was reciprocally crossed with W-38. Ten F_1 plants were cytologically analyzed in each F_1 combination. Except for one plant from a cross of W-38 x No. 2S2, all the F_1s were chimeric. The fact, that No. 2S1, No. 2S2 and the F_1s from crosses with W-38 were chimeric seems to indicate that the chimerism is transmissible or genetic. Thus, to investigate the mode of inheritance of

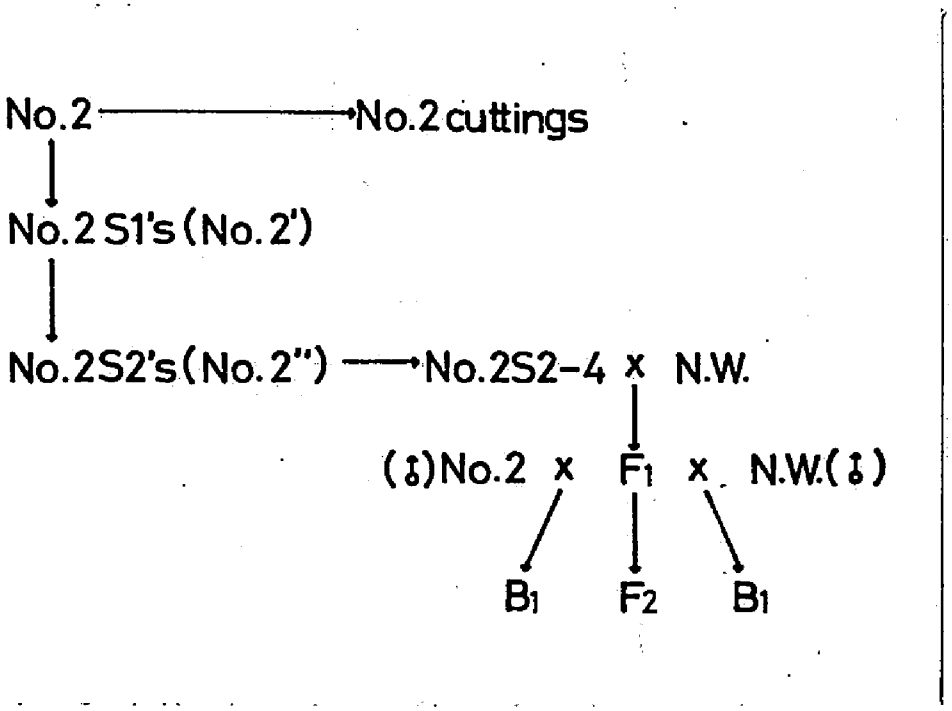


Fig. 16. Diagrammatic representation of the crossing experiment.

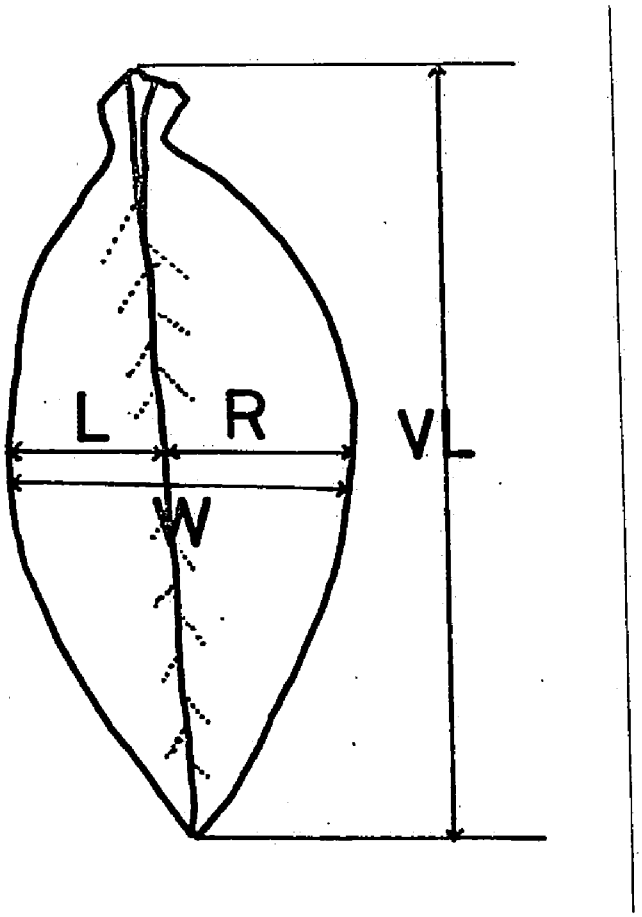


Fig. 17. Schematic representation showing how the measurements on leaf characters were taken.

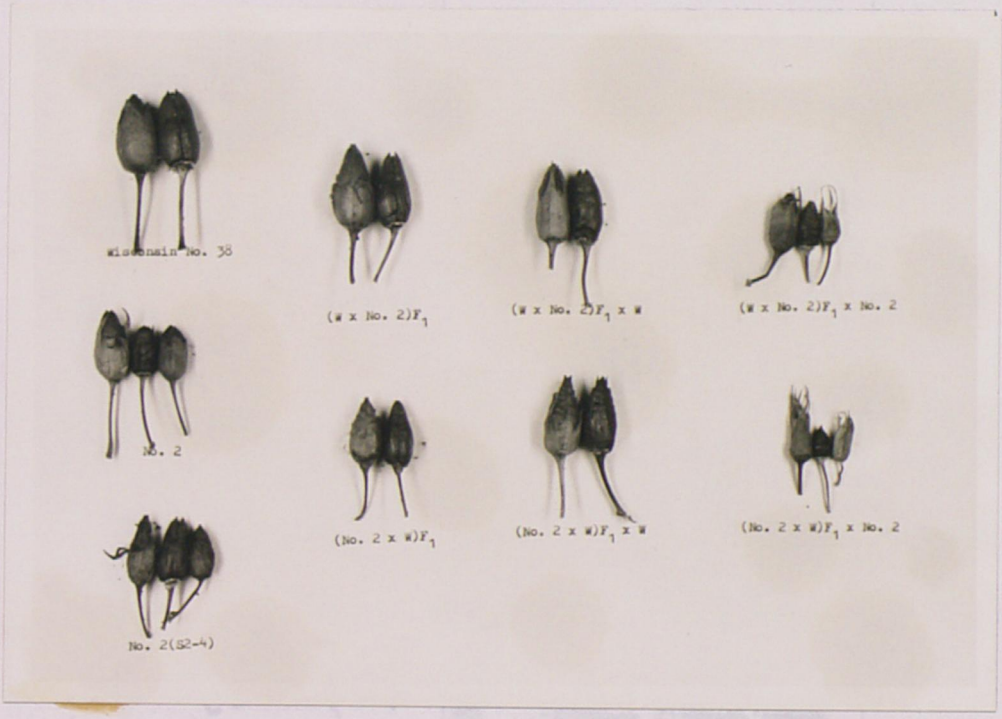


Fig. 18. Capsules of normal Wisconsin No. 38 (W-38), No. 2, No. 2S2-4, F_1s of W-38 x No. 2S2-4 and four B_1s.

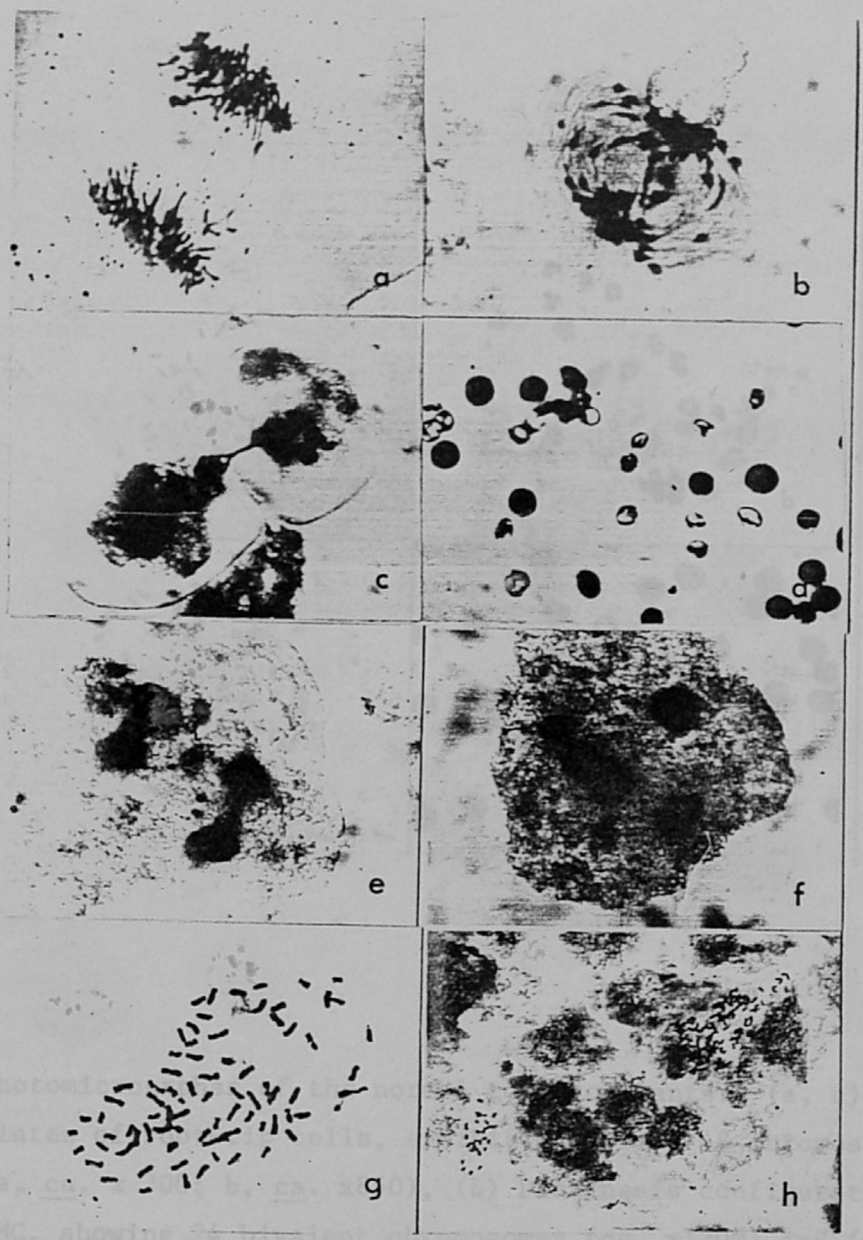


Fig. 19. Photomicrographs showing anomalies of the rooted cuttings of No. 2: (a) Anaphase bridges observed in mitosis (ca. x800), (b) Bridges and laggards in anaphase I of meiosis (ca. x1100), (c) Bridge-like joinings in the pollen dyad stage (ca. x1100), (d) An example of pollen grains (ca. x180), (e) Bridge-like structures in the tetrad stage (ca. x800), (f) Polyad (pentad) formation in the tetrad stage (ca. x800), (g) An aneuploid cell with 78 chromosomes (ca. x1200) and (h) Chromosomal chimerism of root-tip cells within a rooted cutting of No. 2 (ca. x250).

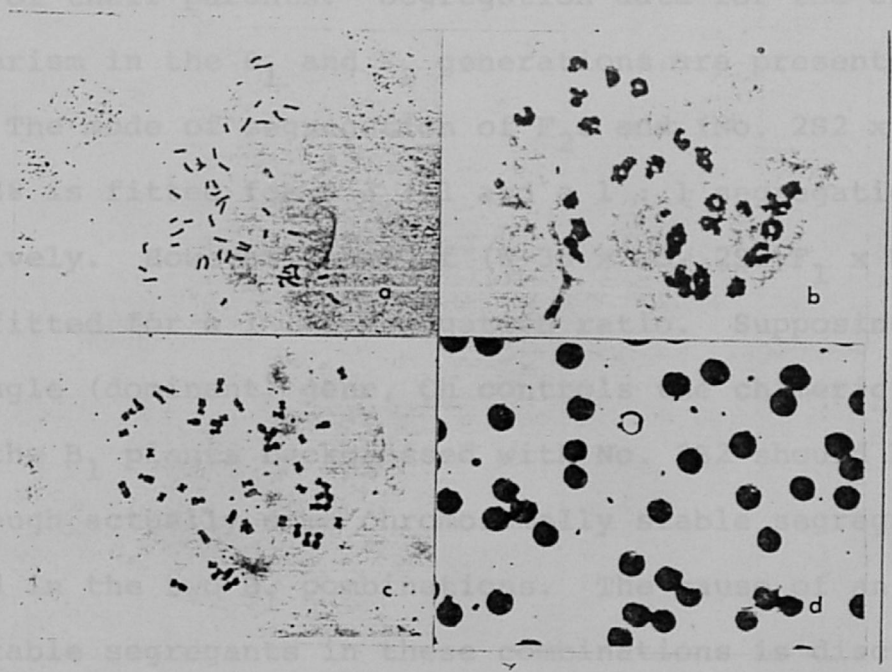


Fig. 20. Photomicrogrphas of the normal tobacco plants: (a, c) Metaphase plates of root-tip cells, exhibiting normal 48 chromosomes (a, ca. x 700; b, ca. x850), (b) Diakinesis configuration of a PMC, showing 24 bivalent chromosomes (ca. x1200) and (d) An example of pollen grains (ca. x200).

the chimerism, F_1s were further selfed and backcrossed with each of their parents. Segregation data for the chromosomal chimerism in the B_1 and F_2 generations are presented in Table 11. The mode of segregation of F_2s and (No. 2S2 x W-38)F_1 x W-38 is fitted for a 3 : 1 and a 1 : 1 segregation, respectively. However, that of (W-38 x No. 2S2)F_1 x W-38 is not fitted for a 1 : 1 segrgation ratio. Supposing that a single (dominant) gene, Ch controls the chimeric character, all the B_1 plants backcrossed with No. 2S2 should be chimeric, although actually some chromosomally stable segregants were found in the two B_1 combinations. The cause of an appearance of stable segregants in these combinations is discussed later.

The data in Table 11 imply that the present chimerism is controlled by a single Mendelian gene (and some modifiers) induced by mutation in tissue culture processes, in which plant growth regulators are involved. Even supposing that a single dominant gene is concerned with the chromosomal chimerism and that it is located in a certain Ch-bearing chromosome (consequently non-chimeric cells) are produced. If the mode of inheritance is a multi- or polygene system, we cannot explain the dominant expression of the chimeric character in the F_1s as well as the comparatively clear segregation in the B_1 and F_2 generations. Another interpretation

Table 11. Segregation of the character chromosomal chimerism in the B_1 and F_2 generations.

Combination	Number of progenies			Ratio (ch:s)	x^2 value
	chimeric	stable	total		
(W-38 x No, 2S2)F_1 x W-38	65	41	106	1.59 : 1	5.43* (1 : 1)
(No. 2S2 x W-38)F_1 x W-38	56	38	94	1.47 : 1	3.44 (1 : 1)
Subtotal	121	79	200	1.53 : 1	8.82**(1 : 1)
(W-38 x No. 2S2)F_1 x No. 2S2	40	7	47	5.71 : 1	
(No. 2S2 x W-38)F_1 x No. 2S2	10	5	15	2.00 : 1	
Subtotal	50	12	62	4.17 : 1	
(W-38 x No. 2S2)F_2	31	10	41	3.10 : 1	0.01 (3 : 1)
(No. 2S2 x W-38)F_2	20	6	26	3.33 : 1	0.05 (3 : 1)
Subtotal	51	16	67	3.20 : 1	0.04 (3 : 1)

* and ** : Significant at the 5% and 1% level, respectively.

is that the chimerism is caused by mixed events in the stem-cell line which may undergo normal cell division and in the mutation-induced altered cell line involved in abnormal cell division, especially as related to spindle anomalies. The ordinary procedures of cytogenetics may not be sufficient, to determine which of the above assumptions is valid for the present chimerism.

Leaf characters of the F_2 and B_1 generations

Fig. 21 shows three leaves of tobacco plants, one from normal W-38 and the others from rooted seedlings of No. 2. It may be noticed in these photographs that the two lines differ from each other with respect to their leaf morphology. Table 12 shows the asymmetry index and foliar ratio in a leaf of tobacco. The data of the line No. 2 were obtained from seven rooted plantlets from the original No. 2. The largest asymmetry index and the smallest foliar ratio was detected in the No. 2 line among eight lines tested (Fig. 22). This indicates that the leaves of No. 2 are most asymmetrical and round. A statistical analysis revealed that the difference of leaf asymmetry between the lines was significant, however, that of foliar ratio was not (Table 13). In the lines, (No. 2S2 x W-38)F_2, (W-38 x No. 2S2)F_1 x W-38 and (No. 2S2 x W-38)F_1 x No. 2S2, all the plants were cytologically checked

Table 12. Instability values of foliar characters in eight lines. (Data indicate the average ± S.E. of seven leaves in each line).

Line	Asymmetry index (x100)	Foliar ratio
W-38	2.2 ± 1.0	2.48 ± 1.02
No. 2	7.6 ± 3.2	1.85 ± 0.94
(W-38 x No. 2S2)F_2	5.5 ± 3.2	1.99 ± 0.82
(No. 2S2 x W-38)F_2	2.9 ± 2.0	2.49 ± 1.03
(W-38 x No. 2S2)F_1 x W-38	4.7 ± 2.2	2.23 ± 0.92
(W-38 x No. 2S2)F_1 x No.2S2	1.8 ± 1.0	2.06 ± 0.85
(No. 2S2 x W-38)F_1 x W-38	3.1 ± 2.0	2.05 ± 0.84
(No. 2S2 x W-38)F_1 x No.2S2	2.7 ± 1.4	2.27 ± 0.94

Table 13. Analysis of variance on two foliar characters.

Source of variation	df	Mean squares	
		Asymmetry index	Foliar rattio
Between lines	7	0.003*	0.25
Within line (Error)	48	0.0007	0.14

* Significant at the 5% level.

Table 14. Analysis of variance on two leaf indices between chimeric and stable tobacco plants.

Source of variation	df	Mean squares	
		Asymmetry index	Foliar ratio
Between lines	1	0.003*	0.358
Within line (Error)	99	0.0007	0.228

* Significant at the 5% level.

Fig. 21. Leaves of normal (left) and the No. 2 lineage (middle and right). Note the marked bilateral asymmetry of leaves of No. 2.

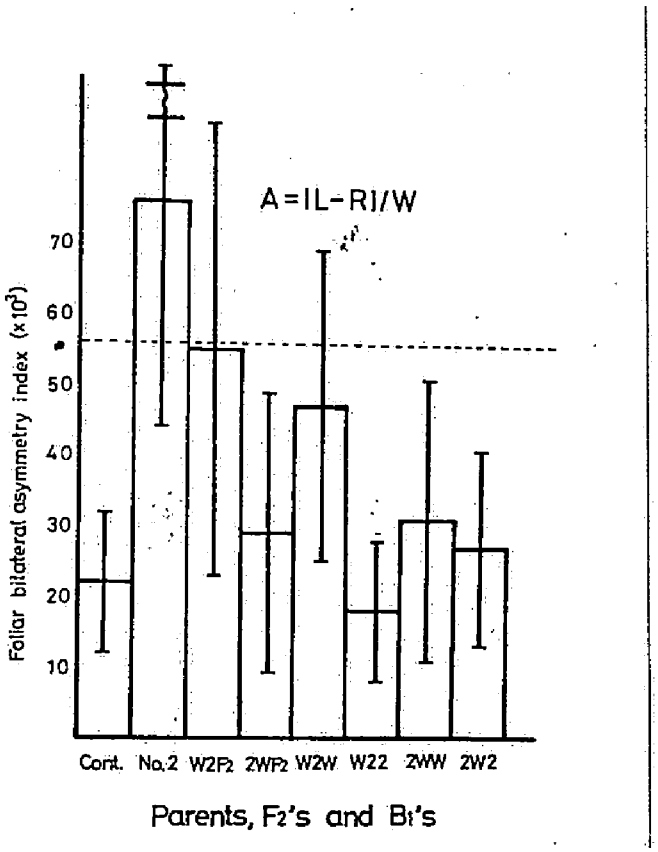


Fig. 22. Magnitude of bilateral asymmetry in the leaves of eight lines of tobacco. Mean ± S.E. of seven individuals in each line (LSD = 0.034).

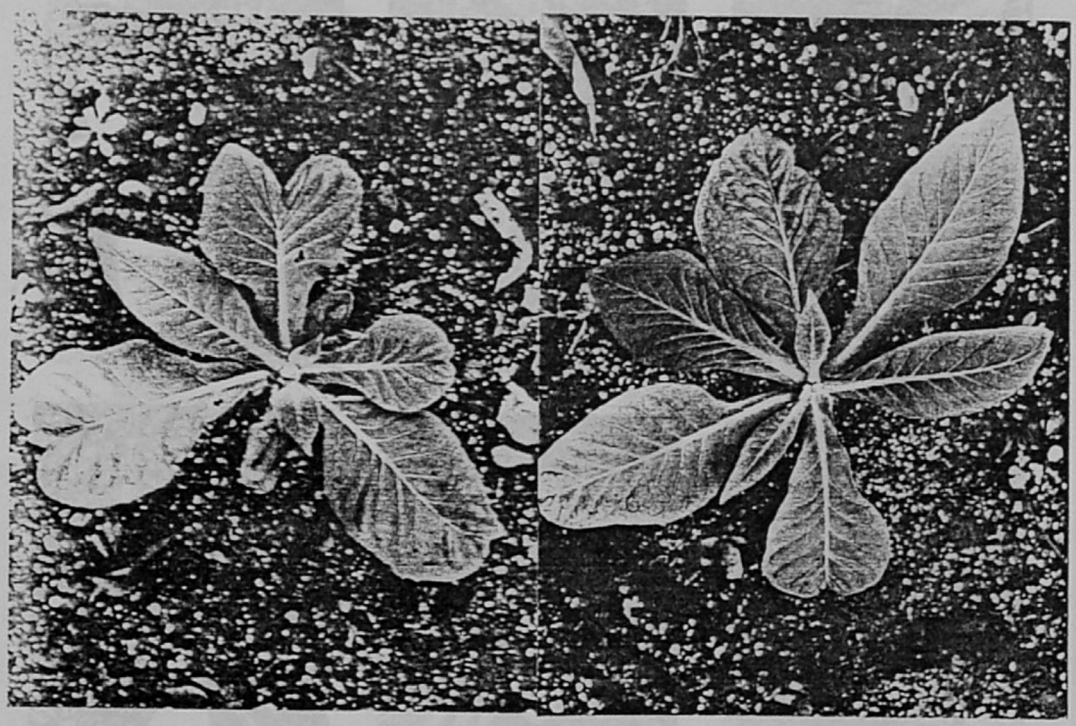


Fig. 23. Tobacco plants showing "chimerism" of leaf forms: left, an individual of (No. 2S2 x W-38)F_2, right, an individual of (No. 2S2 x W-38)F_1 x No. 2 S2.

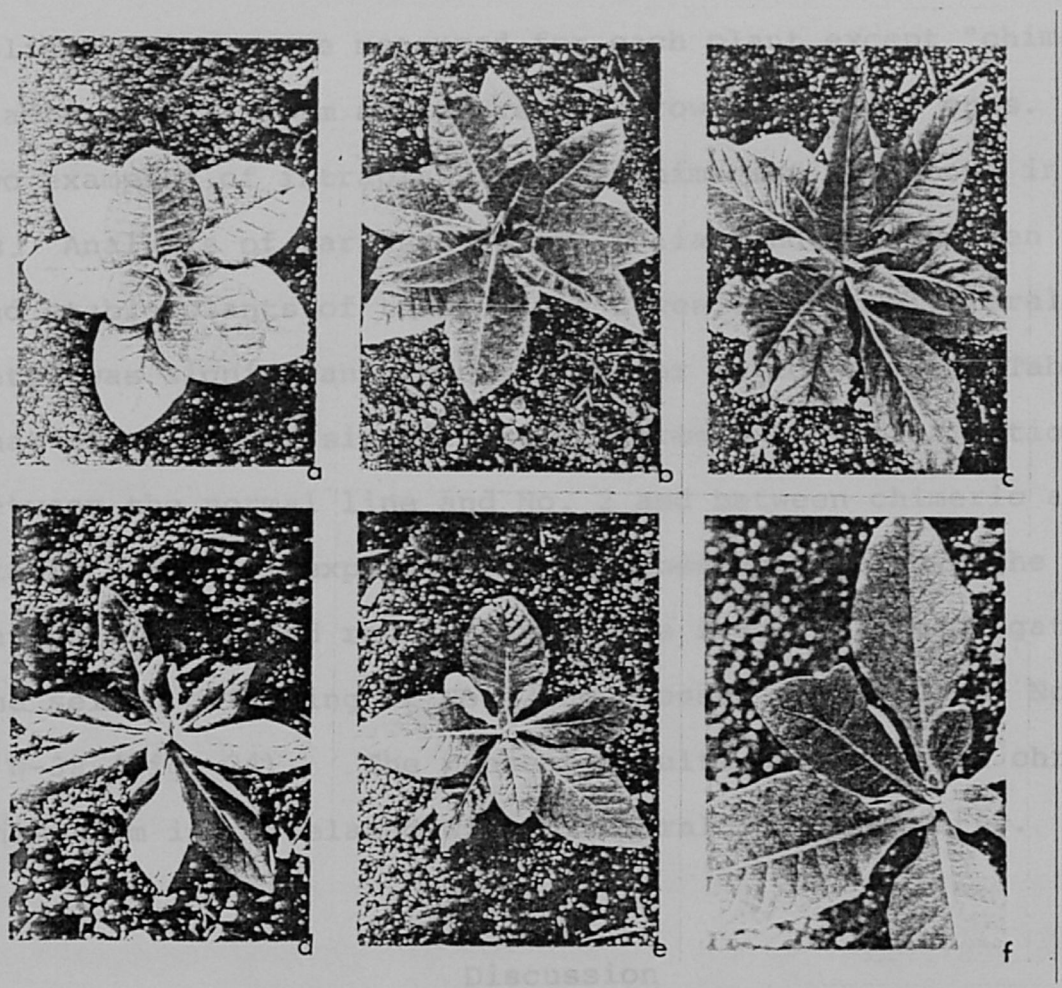


Fig. 24. Several leaf-types in the F_2 and B_1 generations (see text): (a) round, (b) subnormal, (c) cusp-slim, (d) slender, (e) chimeric and (f) diversified venation.

as to chromosomal chimerism. In these three lines, the foliar indices were measured for each plant except "chimeric" plants or leaf form and extremely growth-retarded ones. Two examples of intraplant foliar chimerism are shown in Fig. 23. Analysis of variance on two foliar indices between chimeric and stable plants of these lines revealed that bilateral asymmetry was significant, whereas foliar ratio was not (Table 14). That there was no significant difference in foliar ratio both between the normal line and No. 2 and between chimeric and stable plants is explained by the observations that the plants having slender and round leaves were frequently segregated in the selfed offspring of No. 2 or crossed progenies of No. 2S2 x W-38 (Fig. 24). The present results suggest that chromosomal chimerism is correlated with bilateral leaf asymmetry.

Discussion

Chromosomal chimerism is not a rare phenomenon in higher plants, mainly in their hybrids. A number of investigators have reported the phenomena in somatic tissues (Duncan 1945, Vaarama 1949, Hegwood and Hough 1958, Yang 1965 and others) and in meiosis (Hollingshead 1932, Sachs 1952, Sarvella 1958, Omara 1976 and others). As to the chromosomal constitution

of cultured tissues, there have been two different types of results. One type emphasizes the appearance of aneuploid cells (Straus 1954; de Torok 1960, Norstog et al. 1969, Yamane 1975 and others). and the other shows the majority of callus cells as eudiploid or polyploid (Cooper et al. 1964, Torrey 1967 Butcher et al. 1975 Kodama 1975 and others). In terms of the chromosomal constitution of the regenerated plants from callus cultures, the results of cytological observations are classified into the following two types; the eudiploid or polyploid constitution of original regenerates (Mitra et al. 1960; Blakely and Steward 1964, Venketeswaran and Spiess 1964, Singh et al. 1972, Novak and Vyskot 1975 and others) and the aneuploid consitution of the regenerates (Tabata et al. 1968, Niizeki and Grant 1971, Melchers and Labib 1974, Smith et al. 1976 and others) as well as the somatic chromosomal chimerism of the regenerates (mainly in the Nicotiana and Saccharum species) (Sacristan and Melchers 1969, Heinz et al. 1969, Heinz and Mee 1971, Collins et al. 1972, Speckman and Dijk 1972, Ogura 1975a, 1976, Liu and Chen 1976 and others). The different results may be due to nature of the species used, culture conditions and other known causes. In many cases, the solanaceous species (especially Nicotiana), seem to have a tendency to become variable or unstable in their chromosomal

constitution. As stated, a number of cytological studies have been carried out on the chromosomal constitution of tobacco regenerates, some of which suggest chromosomal chimerism in root-tip cells, and various types of meiotic irregularities in the regenerates (Zagorska et al 1974, Ogura 1975a). Ogura (1976) reported on the chromosomal behavior of the offspring of an original regenerates (designated No. 2). To the knowledge of the author, there have been no reports on the mode of inheritance of chromosomal chimerism. The present study is the first to report the possible existence of a gene(s) involved in chromosomal chimersim which indicates that the mode of inheritance may occur in a simple Mendelian fashion in a regenerate in N. tabacum cv. Wisconsin No. 38. The chimerism found in both the mitosis and meiosis of a regenerate (No. 2) and its offpsring is supported by observations that (1) root cuttings of No. 2 exhibited chimersim both in mitosis and meiosis, (2) the original No. 2 plant was "chimeric" for leaf shape; mixing of round and subnormal leaves was found within the individual, and this trait was transmitted to its offspring and segregated in the B_1 generation when crossed with normal plants, and (3) almost all the F_1s between No. 2 and a normal plant were chimeric and in the F_2 generation, segregation of chimerics vs. stables reasonably fitted a 3 : 1 ratio. In the

B_1 combinations, however, that of (W-38 x No. 2S2)F_1 x W-38 is not fitted for a 1 : 1 segregation ratio. Supposing that a single (dominant) gene, Ch controls the chimeric character, all the B_1 plants backcrossed with No. 2S2 should be chimeric, although actually some chromosomally stable segregants were found in the two B_1 combinations. Since uneaqual separation of chromosomes in the first and second anaphase, probably due to mispairing or other causes occurred in meiosis, whether or not the Ch-bearing chromosome (if a singel Ch is responsible for the chimerism) has entered a given cell in both micro- and megasporogenesis, there is the chance that some gametic combinations whcich do not possess the Ch-bearing chromosome are produced. On this assuption, some stable plants are expected in the offspring of No. 2. The observation of anaphase bridges and laggards (Fig. 19b) in the first anaphase, dyad bridges (Fig. 19c), bridges in tetrad stage (Fig. 19e) and polyad formation (Fig. 19f) in meiosis substatiate this assumption. Therefore, the appearance of some stable segregants in the B_1 generations backcrossed with No. 2S2 is explained. However, in spite of these amonalies observed in meiosis, about half the tetradw were nearly normal in appearance and each monad of each tetrad was nearly equal in size. This suggests that many meiotic cells undergo nearly normal division which finally results in the development of mature

pollen grains, although analysis of the early stages of meiosis was not carried out in detail. Metaphase configurations of PMCs of No. 2 and its rooted cuttings were chimeric for chromosomal constitution, therefore whether these tetrads of nearly normal appearance and of their normal division manner are chromosomally equal is still not known.

Among the various studies on chromosomal chimerism in intact higher plants, some suggested the existence of a gene controlling spindle abnormalities which leads to abnormal cell division in Ribes nigrum (Vaarama 1949), in a variety of common wheat (Watanabe 1962), in diploid hybrids of Solanum tuberosum (Mok and Peloquin 1975a, b), and in Lolium perenne (Omara 1976). For the colchicine-treated Ribes plants, Vaarama (1949) ascribed "chromosomal mosaics" to the altered genic control induced by the permanent effect of colchicine. Watqnabe (1962) assumed a gene inducing spindle abnormalities, although he added that detailed study of the processes in the early stages before diakinesis is required to determine the certain existence of such a gene. Mok and Peloquin (1975a, b) reported three mechanisms (spindle abnormalities and two types of premature cytokinesis) of 2n pollen (diplandroid) formation in diploid potatoes, and based on the results of test crosses and allelism tests, suggested that the three mechanisms of diplandroid formation

are controlled by simple recessive genes induced by mutation. Omara (1976) recently reported chromosomal chimerism of PMCs in diploid rye-grasses due to cytomixis or the mechanism for for chromatin passage from one PMC to an adjacent PMC. However, the cause of cytomixis is still obscure. Based on the segregation data presented in Table 1 in this study, the present chromosomal chimerism in a tobacco regenerate and its offspring was interpreted by assuming that a simple dominant gene, presumably induced in the processes of tissue culture, is responsible for the phenomenon. Otherwise, the chimerism may be caused by indipendent events in the normal cell line and by a mutation-induced altered cell line in a plant; the latter being involved in abnormal cell division. According to Makino (1957), certain animal and plant tissues contain several cell populations, each with different chromosome numbers. Some of these different cell populations could be separated by callus formation in vitro, whereby complete plants are regenerated. Consequently, the derived plants would each have a different chromosome number. This stemline theory could partly esplain the existence of callus derivatives which differ genetically from the original normal tobacco plants. It should also be noted that the present chimeric regenerate (No. 2) was induced in the medium containing a combination of KIN and a

morphactin, CFM. Chromosomally chimeric regenerates were sometimes obtained with a combination of IAA and KIN or by a single addition of KIN in the medium, although almost all the selfed progenies of such regenerates stabilized to a certain chromosome number (Ogura, unpublished). Morphactins are a group of compounds originally synthesized in the Research Laboratories of E. Merck, AG, Darmstadt, Germany. Schneider (1964) first described the biological effects of these substances on plant morphogenesis and reviewed their general actions on plants (Schneider 1970). Among their various effects, particularly those on cell division and chromosomal behavior, are the reduction of the number of mitoses in onion root-tip cells (Denffer et al. 1969), a change in the orientation of the spindle axis of the dividing cells of pea root-tips (Ziegler 1970) and partial desynapsis of chromosomes in the first metaphase of meiosis in Nicotiana sylvestris plants reared from CFM-soaked seeds (Ogura, in prep.). Considering these influences of morphactins on cell division and chromosomal behavior, we cannot ignore the effects of CFM on chromosomal chimerism. Cytogenetically and physiologically nearly normal plantlets were also regenerated from the medium containing CFM and KIN, therefore the presence of CFM in the medium does not always seem to be the sole cause of chromosomal chimerism. As to

leaf characters, Sakai and Shimamoto (1965) suggested that the bilateral leaf asymmmetry of tobacco is under genetic control. In the present study, the bilateral leaf asymmetry is mainly related to chromosomal chimerism. The effects of morphactins on cell division and leaf morphology, as well as the cytological analyses of chromosomal constitution of regenerates derived from cultured tissues should be further investigated.

Summary

As described in Chapter 5, a chromosomally chimeric line (No. 2) was found. A progeny plant of No. 2, No. 2S2-4 was reciprocally crossed with the normal Wisconsin No. 38 plant (W-38). All the F_1s were chimeric except one plant in (W-38 x No. 2S2)F_1 combination. Both F_1s were further crossed with each of their parents. On the basis of segregation data in F_2s and four B_1s, the mode of inheritance can be interpreted by assuming that the chimerism is mainly controlled by a simple Mendelian gene induced during tissue culture by growth regulators. Inheritance of two foliar characters, namely bilateral asymmetry and foliar ratio was also studied in relation to the chromosomal chimerism. The

chromosomally unstable No. 2 line showed larger than the normal line, however, no significant difference was found on the difference between chimeric and stable plant plants pooled from three lines was significant in the bilateral asymmetry, but not in foliar ratio. The results suggest that bilateral leaf asymmetry of tobacco plants is correlated with the chromosomal chimerism.

Chapter 7. DIFFERENTIAL RESPONSES OF NICOTIANA TABACUM L. AND ITS PUTATIVE PROGENITORS TO DE- AND REDIFFERENTIATION

Introduction

Skoog and Miller (1957) demonstrated that pith-derived calluses of Nicotiana tabacum L. cv. Wisconsisn No. 38 could be induced to redifferentiate into roots or shoots, depending on the relative amount of IAA and KIN added in the medium. Similar interactions of IAA and various kinds of cytokinins on growth and organ redifferentiation from tobacco pith callus have also been reported (Hamzi and Skoog 1964, Vasil and Hildebrandt 1967, Skoog 1970). Kochhar et al. (1970, 1971) have indicated that several tobacco smoke carcinogens can exert the morphogenetic effect like the combinations of IAA and KIN on the pith-derived calluses of haploid tobacco plant, but not of normal diploid. Recently, Ogura (1975) has observed that some combinations of KIN and CFM result in 100% shoot redifferentiation in inoculated tobacco calluses.

Yet, few literatures are available on the relationships between the differences of genetic constitution and callus formation or organ redifferentiation. The involvements of

genomic differences with callus induction in Triticum-Aegilops group have been considered (Ogura 1977). The author here reports the differential responses of callus induction and shoot redifferentiation among a cultivar of N. tabacum, its putative progenitos, N. sylvestris and N. tomentosiformis, an artificially synthesized amphiploid from them, and some other species of the genus Nicotiana.

Materials and Methods

Dedifferentiation (callus induction)

Several tissues and organs taken from Nicotiana tabacum L. cv. Wisoconsin No. 38 (W-38), N. sylvestris Speg. and Comes, N. tomentosiformis Goodsp., N. tomentosa Ruiz and Pavon, N. otophora Grieseb. and an amphiploid synthesized from N. sylvestris and N. tomentosiformis, that was originally produced by Dr. L. G. Burk (Burk 1973), and is designated as "Burk's F_5", were aseptically explanted on an RM-1964 basal medium (Linsmaier and Skoog 1965) containing three different concentrations of IAA, KIN and 2,4-D. The media used for callus induction were as follows:

Medium A: RM-1964 + 3.0 mg/l IAA + 0.2 mg/l KIN

Medium B: RM-1964 + 0.1 mg/l IAA

Medium C: RM-1964 + 6.0 mg/l IAA + 5.0 mg/l 2,4-D

The pH of all the media was adjusted to 5.8 ± 0.1 before autoclaving. The esperiments were acrried out at 25 ± 1°C in the dark throughout.

Redifferentiation (shoot formation)

Induced calluses of W-38, Burk's F_5 and N. sylvestris were subcultured in the same medium (medium A). After about 30 days of subculturing, vigorously proliferating calluses were aseptically cut into small pieces by a scalpel and the pieces (ca. 200 mg each) were inoculated to test-tubes containing an RM-1964 basal medium added with 2.0 mg/l of KIN and 1.0 mg/l of CFM, as previously described by Ogura (1975b, c).

Induced calluses of N. tomentosiformis and N. otophora were directly used for a shoot redifferentiation experiment, since the growth of N. tomentosiformsis calluses originated from peduncle (Fig. 26) was very slow, but that of N. otophora was not so slow. Therefore, only five pieces of N. otophora (ca. 200 mg each) were inoculated to the same shoot regeneration medium as used for Burk's F_5 and N. sylvestris.

Results

Callus induction

Pith discs or seedling roots of W-38, N. sylvestris

Fig. 26. Callus of N. tomentosiformis induced from excised peduncles (left), N. otophora callus from immature capsule (middle) and N. tabacum callus of pith origin, after 60 days of culture (ca. x 1.4). Note the difference of callus growth among these three species.

and Burk's F_5 formed calluses after 30 days of culture, when tissues or seeds were inoculated into medium A. In contrast, those of N. tomentosiformis, N. tomentosa and N. otophora, all of which belong to the section Tomentosae, formed no calluses in the same medium. The results are summarized in Table 16. In medium B, escised tissues or organs of the three species of the Tomentosae section produced no calluses. Several organs or tissues of N. tomentosiformis and N. otophora were also esplanted in medium C of high auxin level. After about 40 days of culture, most tissues of N. otophora formed calluses; but only the flower peduncle explants of N. tomentosiformis showed callus formation after about 60 days of culture (Table 17 and Fig. 26). Calluses of W-38, Burk's F_5 and N. sylvestris (in medium A) were brownish-white and comparatively vigorous and uniform growth, those of N. otophora (in medium C) were dark brown and grew comparatively vigorously, and those of N. tomentosiformis (in medium C) were light brown and showed the least growth (Fig. 26). The data indicate roughly the following order of ease of callus formation: N. tabacum (W-38) Burk's F_5 N. sylvestris N. otophora N. tomentosiformis.

Shoot redifferentiation

Table 18 shows the results of shoot redifferentiation experiments after about 50 days of culture: W-38 calluses of

Table 16. Degree of callus formation and callus growth in several Nicotiana species in the medium A. Data were taken after about 30 days of culture.

Species	Genome formula	Organ or tissue explanted	Degree of callus formation	Callus growth
tabacum Wisconsin No. 38	SSTT	pith discs	++	++
		roots	++	++
sylvestris	SS	pith discs	±	±
		roots	+	+
tomentosiformis	TT	pith discs	-	
		roots	-	
tomentosa	TT	pith discs	-	
		roots	-	
otophora	TT	pith discs	-	
		roots	-	
rk's F_5 (N. sylvestris x N. tomentosiformis)	SSTT	pith discs	+	+
		roots	+	+

te) Pith discs used are those excised from nearly mature plants. Roots shows those from young seedling roots grown from seeds.

Degree of callus formation: - no formation, ± occasional formation, + frequent formation, ++ consistent formation.
Callus growth: ± —— ++, weak to vigorous growth in various degrees.

Table 17. Callus formation of several different explants from two Tomentosae species in the medium C, after 60 days of culture.

Species	Organ or tissue explanted	Degree of callus formation
N. tomentosiformis	pith	-
	stem	-
	leaf-midvein	-
	peduncle	±
	flower bud	-
	anther	-
	immature capsule	-
N. otophora	pith	±
	stem	+
	peduncle	+
	anther	±
	immature capsule	+

Callus formation: -, no formation; ±, occasional formation; +, frequent formation; ++, consistent formation.

Table 18. Shoot redifferentiation from calluses of several *Nicotiana* species.

Species	Genome formula	No. calluses inoculated	No. calluses with shoots	%
N. tabacum (W-38)	SSTT	20	20	100
Burk's F_5	SSTT	10	8	80
N. sylvestris	SS	10	10	100
N. tomentosiformis	TT	5	0	0
N. otophora	TT	10	0	0

both pith and seedling root origin and N. sylvestris calluses of seedling root origin consistently exhibited the highest (loo%) shoot redifferentiation rate. Burk's F_5 calluses of pith origin resulted in relatively high shoot formation. However, both N. tomentosiformis and N. otophora calluses showed no shoot regeneration. Fig. 27 shows an example of calluses of several Nicotiana species in the shoot redifferentiation medium after 50 days of culture.

Discussion

To the knowledge of the author, this is the first attempt to induce calluses of N. tomentosiformis and N. otophora, although Collins et al. (1972) obtained haploid plants from N. otophora by anther culture in very low frequency. The requirement of high auxin level to induce callus formation in N. tomentosiformis and N. otophora suggests this might be common to species of the section Tomentosae, although suitable conditions for callus induction and subculturing of the callus should be further. The present results show the medium for callus induction may be one of the following two types: a standard tobacco medium (medium A) for N. tabacum, a synthesized amphiploid strain (Burk's F_5) and N. sylvestris; and a medium

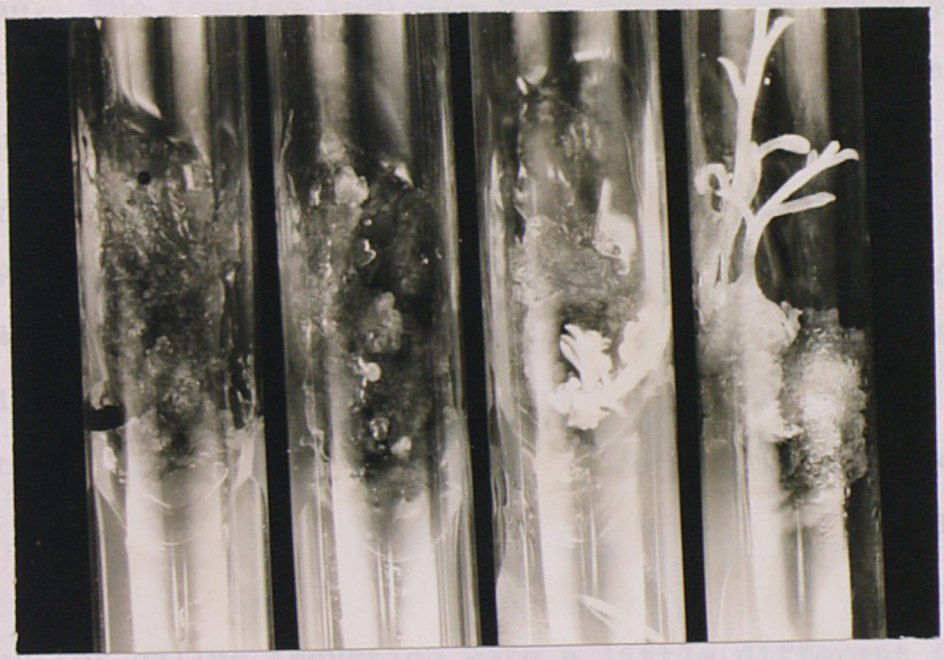


Fig. 27. Differential responses of four kinds of Nicotiana calluses to the shoot redifferentiation medium after 50 days of culture. From left to right: Calluses of N. tomentosiformis, N. otophora, Burk's F_5 (an amphiploid synthesized from N. sylvestris and N. tomentosiformis) and N. tabacum cv. Wisconsin No. 38 (ca. x 1.2).

containing a high auxin level of a synthetic auxin, 2,4-D (medium C) for N. tomentosiformis and N. otophora. The results also indicated that peduncle parts are suitable for callus induction in the present two species of the section Tomentosae. The results indicate that these Tomentosae plants having T-genome require higher auxin concentration for callus formation than cultivated tobacco, ST amphiploid (Burk's F_5) and N. sylvestris.

As presented in Table 18, calluses of W-38 and N. sylvestris gave 100% shoot formation in a 50-day culture, and Burk's F_5 callus also gave relatively high shoot formation (80%). However, both N. tomentosiformis and N. otophora calluses exhibited no shoot formation. The same shoot redifferentiation medium was used for all kinds of the Nicotiana calluses, although the conditions for callus induction were a little different for N. tabacum, N. sylvestris and Burk's F_5 and for the two Tomentosae species. The data in Table 16 imply that genetic (and genomic) factors involved in shoot redifferentiation in N. tabacum and its synthetics are mainly derived from their S genome donor, a progenitor or relative of N. sylvestris. The difference in the results on callus induction, growth and shoot redifferentiation of the callus between N. tabacum W-38 and Burk's F_5 may be due to a difference in their genetic background. According to available evidence, N. tabacum (2n=48, genome formula SSTT)

is assumed to be amphiploid arising from chromosome doubling following hybridization between a progenitor of N. sylvestris (an=24, SS) and an ancestral type similar to, but not identical with, N. tomentosiformis (2n=24, TT). Genetic studies by Gerstel (1960) and cytogenetic investigations by Clausen (1932), Kostoff (1938) and Cameron (1965), and biochemical studies on isozyme patterns by Sheen (1972) and on Fraction I protein by Kung et al. (1975), as well as the production of a fertile amphiploid by Burk (1973) from in vitro callus culture of the sterile F_1 hybrid between N. sylvestris and N. tomentosiformis, support this assumption.

As for dedifferentiation, Tabata and Motoyoshi (1963) reported that in maize endosperm culture, the endosperm explants of only a specific genotype (starchy) formed calluses, whereas those of other genotypes did not. The genetic role of individual chromosomes or chromosome arms in callus formation have been reported in anther culture of A-genome aneuploid of common wheat (Shimada and Makino 1975). Cytoplasm effects on callus induction from wheat anthers have been reported, from studies on the cytoplasm substitution lines of Chinese Spring wheat (Ogura and Tsunewaki 1974). Recently, Ogura (1977) has indicated that there exists some genetical differences between polyploids in wheat on callus induction, suggesting

that the feasibility of callus formation in wheat seedling roots cultured in vitro is in the following order: diploid (genome formula AA), tetraploid (AABB) and hexaploid (AABBDD).

The present work first showed a possible location of the genetic factors in a specified genome for shoot redifferentiation in the genus Nicotiana. From the data given in Tables 14 and 16, the feasibility of callus formation seem to parallel with that of shoot redifferentiation to a considerable extent. The author compared the degree of rooting of cuttings between N. tabacum W-38, N. sylvestris and N. tomentosiformis on a small scale (Ogura unpublished). The results suggested that the feasibility of vegetative propagation by the rooting of shooots from axilllary buds or stem tips is not necessarily related to that of shoot redifferentiation or of callus formation. It is yet to be established species belonging to the section Tomentosae present difficulties in induction of callus formation and in redifferentiation into shoots. Here, we considered the relationships between genomic differences and tissue culture properties in several Nicotiana species; studies of this line should be pursued further on a more general scale.

Summary

Calluses of N. tabacum, N. sylvestris, N. tomentosiformis

N. otophora and an artificially synthesized amphiploid between N. sylvestris and N. tomentosiformis (Burk's F_5) were induced. For induction and subculturing of calluses of N. tabacum, N. sylvestris amd Burk's F_5, an RM-1964 basal medium containing 3.0 mg/l of IAA and 0.2 mg/l of KIN as described by Skoog and Miller (1957) was used. In the case of callus induction in N. tomentosiformis and N. otophora, however, an RM-1964 medium of far higher auxin level as compared with the above-mentioned three species was required. Induced calluses of these species were inoculated in the identical shoot redifferentiation medium, which was successful for 100% shoot redifferentiation in inoculated calluses, at least in N. tabacum and N. sylvestris. All of N. tabacum and N. sylvestris calluses inoculated redifferentiated shoots, whereas N. tomentosiformis and N. otophora calluses resulted in no shoot formation. The results seem to indicate that genetic factors involved in shoot redifferentiation in N. tabacum are localized in the N. sylvestris genome.

Chapter 8. GENERAL CONCLUSION

Some combination of a morphactin, chlorflurenol-methyl-ester (CFM) and kinetin (KIN) in appropriate concentrations exerted more efficient influences on shoot organogenesis from tobacco callus as compared with the known kinetin-auxin combination. However, by applying CFM, in combination with KIN, more teratoma regenerates were obtained than by a single application of KIN to the medium. Almost all the plantlets regenerated by any kind of media exhibited, more or less, chromosomal chimeras. A plant showed chromosomal chimerism through three generations and the existence of simple gene(s) involved in chromosomal chimerism was substatiated. The chromosomal chimerism was supported by the facts that cytological irregularities both in mitosis and meiosis such as anaphase bridges were found, and that intraplant chimerism of leaf forms was observed. There was a significant correlation between chromosomal chimerism and foliar bilateral asymmetry. Thus, the possibility of plant tissue culture as a potential tool to obtain variants or mutants was demonstrated. The feasibility of shoot redifferentiation from calluses were found to be different among various species of Nicotiana. A probable location of the genetic factors controlling regenerative ability from callus was first specified into a certain genome.

The findings were itemized as follows:

(1) Some combinations of KIN and CFM resulted in 100% shoot redifferentiation in inoculated calluses.

(2) CFM acted as a growth retardant in cultured tissues, but in the presence of KIN, it counteracted with KIN and acted stimulatory for shoot redifferentiation.

(3) Irrespective of the kinds or combinations of growth regulators used for shoot organogenesis, almost all the original regenerates exhibited chromosomal chimerism.

(4) After selfing, most of the regenerates stabilized to have a certain chromosome number (eudiploid, polyploid or aneuploid).

(5) A lineage of chromosomally chimeric strain through three generations was obtained from the medium containing 1.0 mg/l of CFM and 2.0 mg/l of KIN.

(6) Crosses were made reciprocally between this chimeric strain and normal plants and the F_1, F_2 and B_1 generations were studied. Based on the segregation data from B_1s and F_2s, the chimeric character was considered to be mainly controlled by a single dominant gene induced by mutation during tissue culture.

(7) Some foliar characters of No. 2, normal tobacco and their B_1s and F_2s were recorded, and based on the data of the bilateral

asymmetry in the leaf and of the ratio of the latitudinal to logitudinal length, instability values were calculated. The results suggested that the magnitude of chromosomal chimerism and instability of foliar characters were positively correlated.

(8) Chromosomal chimerism paralleled with other characters, such as pollen and seed fertilities, dwarfism, leaf form, abnormal inflorescence shape etc.

(9) Genetic factor(s) involved in shoot redifferentiation in N. tabacum and its synthetics (Burk's amphidiploid) are probably located in their N. sylvestris genome.

ACKNOWLEDGEMENT

The author wishes to thank to Dr. K. Tsunewaki, Professor of the Laboratory of Genetics, Kyoto University for his guidance and critical reading of the manuscript.

He is indebted to Dr. M. Tanaka, Professor of Kyoto University for his valuable suggestions and interest to the present work.

The author thanks Dr. H. Ono, Associate Professor of Kobe Univeristy for his helpful suggestions to the early part of this work, Dr. L. G. Burk, Professor of North Carolina State University for hias generously supplying the synthetic amphiploid lines of Nicotiana tabacum and Messrs. S. Tsuji T. Koba of Kyoto University for their reliable technical assistances.

The author is grateful to Japan Tobacco & Salt Public Corporation for supplying the tobacco seeds used and the permission of cultivating tobacco plants. The morphactin was generously supplied to Dr. K. Tsunewaki by Kyowa Hakko Kogyo Co. Ltd., Tokyo.

LITERATURE CITED

Bergmann, L. 1960 Growth and division of single cells of higher plants in vitro. J. Gen. Physiol. 43: 841-851.

Blakely, L. M. and F. C. Steward. 1964 Growth and organized development of cultured cells. VII. Cellular variation. Amer. J. Bot. 51: 809-820.

Bonner, J. and F. Addicott. 1937 Cultivation in vitro of excised pea roots. Bot. Gaz. 99: 144-170.

Bopp, M. 1969 Die Wirkung von Morphaktinen auf die Streckung von Sprossachsen. In "Symposium Morphaktine, Vortraege aus dem Gesamtgebiet der Botanik Neue Folge 3" Ed. G. Mohr and H. Ziegler pp. 69-76. G. Fischer Verlag, Stuttgart.

Bourgin, J. P. and J. P. Nitsch. 1967 Obtention de Nicotiana haploide a partir d'etamines cultivees in vitro. Ann. Physiol. veg. 9: 377-382.

Burk, L. G. 1973 Partial self-fertility in a theoretical amphiploid progenitor of N. tabacum. J. Hered. 64: 348-350.

Butcher, D. N., A. K. Sogeke and I. C. Tommerup. 1975 Factors influencing changes in ploidy and nuclear DNA levels in cells from normal, crown-gall and habituated cultures of Helianthus annus L. Protoplasma 86: 295-308.

Caplin, S. M. and F. C. Steward. 1949 A technique for the controlled growth of excised plant tissue in liquid media under aseptic conditions. Nature 163: 920-924.

Cameron, D. R. 1965 Cytoplasmic effects in Nicotiana. Proc. 11th Intern. Congr. Genet. 1: 203-204.

Carlson, P. S. 1969 Production of auxotrophic mutants in ferns. Genet. Res. 14: 337-339.

Carlson, P. S. 1970 Induction and isolation of auxotrophic mutants in somatic cell cultures of Nicotiana tabacum. Science 168: 487-489.

Carlson, P. S. 1973 The use of protoplasts for genetic research. Proc. Natl. Acad. Sci. U.S.A. 70: 598-602.

Carlson, P. S., H. H. Smith and R. D. Dearing. 1972 Parasexual interspecific plant hybridization. Ibid. 69: 2292-2294.

Clausen, R. E. 1932 Interspecific hybridization in Nicotiana. XII. Further data as to the origin of N. tabacum. Svensk. Bot. Tidskn. 26: 123-126.

Cocking, E. C. 1960 A method for the isolation of plant protoplasts and vacuoles. Nature 187: 927-929.

Cocking. E. C. 1966 An electron microscopic study of the initial stages of infection of isolated tomato fruit protoplasts by tobacco mosaic virus. Planta 68: 206-214.

Collins, G. B., P. D. Legg and M. J. Kasperbauer. 1972 Chromosome numbers in anther-derived haploids of two Nicotiana species. J. Hered. 63: 113-118.

Cooper, L. S., D. C. Cooper, A. C. Hildebrandt and A. J. Riker. 1964 Chromosome numbers in single cell clones of tobacco tissue. Amer. J. Bot. 51: 284-290.

Denffer, D. von, Fricke, G. and F. Ringe. 1969 Durch 2-Chlor-9-hydroxyfluorencarbonsäure-(9) hervorgerufene morphogenetische Effekte bei Stypocaulon scoparium, Begonia x richmondensis und Wurzelspitzen von Allium cepa. Ber. Dtsch. Bot. Ges. N. F. 3: 61-68.

de Ropp, R. S. 1955 The growth and behavior in vitro of isolated plant cells. Proc. Roy. Soc. (Lond.) B 144: 86-93.

de Torok, D. and P. R. White. 1960 Cytological instability in tumors of Picea glauca. Science 131: 730-732.

Doy, C. H., P. M. Gresshoff and B. G. Rolfe. 1973 Biological and molecular evidence for the transgenosis of genes from bacteria to plant cells. Proc. Natl. Acad. Sci. U.S.A. 70: 723-726.

Dulieu, H. 1972 The combination of cell and tissue culture with mutagenesis for the induction and isolation of morphological or developmental mutants. Phytomorph. 22: 283-296.

Duncan, R. E. 1945 Production of variable aneuploid numbers of chromosomes within the root-tips of Paphiopedium wardii. Amer. J. Bot. 32: 506-509.

Fox, J. E. 1963 Growth factor requirements and chromosome number in tobacco tissue cultures. Physiol. Plant. 16: 793-803.

Fukumoto, K. 1962 Nuclear instability and chromosomal mosaicism in higher polyploids of Solanum species and hybrids. Japan. J. Bot. 18: 19-53.

Gamborg, O. L., F. Constabel, L. Fowke, K. N. Kao, K. Ohyama, K. Kartha and L. Pelcher. 1974 Protoplast and cell culture methods in somatic hybridization in higher plants. Can. J. Genet. Cytol. 16: 737-750.

Gautheret, R. J. 1934 Culture de tissu cambial. C. R. hebd. Seanc. Acad. Sci., Paris 198: 2195-2196.

Gautheret, R. J. 1939 Sur la possibilite de realiser la culture indefinie des tissu du tubercules de carotte. Ibid. 208: 118-121.

Gautheret, R. J. 1955 The nutrition of plant tissue cultures. Ann. Rev. Plant Physiol. 6: 433-484.

Gerstel, D. U. 1960 Segregation in new allopolyploids of Nicotiana. I. Comparison of 6x (N. tabacum x N. tomentosiformis) and 6x (N. tabacum x N. otophora). Genetics 45: 1723-1734.

Gerstel. D. U. and J. A. Burns. 1966 Flower variegation in hybrids between Nicotiana tabacum and N. otophora. Genetics 53: 551-567.

Giaja, J. 1919 Cited from E. C. Cocking, 1972 Plant cell protoplasts- isolation and development. Ann. Rev. Plant Physiol. 23: 29-50.

Guha, S. and S. C. Maheshwari. 1966 In vitro production of embryos from anthers of Datura. Nature 204: 497.

Guha, S. and S. C. Maheshwari. 1967 Cell division and differentiation of embryos in the pollen grains of Datura in vitro. Nature 212: 97-98.

Guha, S. and S. C. Maheshwari. 1974 Development of embryoids from pollen grains of Datura in vitro. Phytomorph. 17: 454-461.

Haberlandt, G. 1902 Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber. Akad. der Wiss. Wien, Math-Naturwiss. 111: 69-92.

Haccius, B. 1969 Anomalien der pflanzlichen Embryogenese nach Einwirkung von 2,4-D, TIBA und morphaktin, ein Vergleich. In "Symposium Morphaktine" Ed. G. Mohr und H. Ziegler. Ber. Dtsch. Bot. Ges. N.F. 3: 89-101.

Hamzi, H. Q. and F. Skoog. 1964 Kinetin-like growth promoting activity of 1-substituted adenines (1-benzyl-6-aminopurine and 1-(gamma, gammadimethylallyl)-6-aminopurine). Proc. Natl. Acad. Sci. U.S.A. 51: 76-83.

Hegwood, M. P. and L. F. Hough. 1958 A mosaic pattern of chromosome numbers in the white winter pearmain and six of its seedlings. Amer. J. Bot. 45: 349-354.

Heinz, D. J. and G. W. P. Mee. 1971 Morphological, cytogenetic and enzymatic variation in Saccharum species hybrid clones derived from callus tissue. Amer. J. Bot. 58: 258-262.

Heinz, D. J., G. W. P. Mee and L. G. Nickell. 1969 Chromosome numbers of some Saccharum species hybrids and their cell suspension cultures. Amer. J. Bot. 56: 450-456.

Hildebrandt, A. C., A. J. Riker and B. M. Duggar. 1946 The influence of the composition of the medium on growth in vitro of excised tobacco and sunflower tissue cultures. Amer. J. Bot. 33: 591-597.

Hollingshead, L. 1932 The occurrence of unpaired chromosomes in hybrids between varieties of Triticum vulgare. Cytologia 3: 119-141.

Jones, L. E., A. C. Hildebrandt, A. J. Riker and J. H. Wu. 1960 Growth of somatic tobacco cells in microculture. Amer. J. Bot. 47: 468-475.

Kao, K. N. and M. R. Michayluk. 1974 A method for high-frequency intergeneric fusion of plant protoplasts. Planta 115: 355-367.

Kao, K. N., F. Constabel, M. R. Michayluk and O. L. Gamborg. 1974 Plant protoplast fusion and intergeneric hybrid cells. Planta 120: 215-227.

Kochhar, T. S., P. R. Bhalla and P. S. Subharwal. 1970 In vitro induction of vegetative buds by benz(a)anthracene in tobacco callus. Planta 94: 246-249.

Kochhar, T. S., P. R. Bhalla and P. S. Subharwal. 1971 Effects of tobacco smoke components on organogenesis in plant tissues. Plant & Cell Physiol. 12: 603-608.

Kodama, A. 1975 Karyological studies on crown gall tumors of the pea. Japan. J. Genet. 50: 291-299.

Kostoff, D. 1938 Studies on polyploid plants. XVIII. Cytogenetic studies on Nicotiana sylvestris x N. tomentosiformis hybrids and amphidiploids and their bearings on the problem of the origin of N. tabacum. C. R. Acad. Sci. U. R. S. S. 18: 459-462.

Kotte, W. 1922 Kulturvesuche mit isolierten Wurzelspitzen. Beitr. Allg. Bot. 2: 413-434.

Krelle, E. 1967 Untersuchungen ueber die Wirkung des "Anti-gibberellins", 2-chloro-9-flurenol-(9)-carbonsaure-methyl-ester. Wiss. Z. Univ. Rostock, Math.-Naturwiss. 16: 691-692.

Kung, S. D., J. C. Gray, S. G. Wildman and P. S. Carlson. 1975 Polypeptide composition of fraction I protein from parasexual hybrid plant in the genus Nicotiana. Science 187: 353-355.

Ledoux, L. and R. Huart. 1971 Fate of exogeneous DNA in Arabidopsis thaliana. Eur. J. Biochem. 23: 96-108.

Liu, M. C. and W. H. Chen. 1976 Tissue and cell culture as aids to sugarcane breeding. I. Creation of genetic variation through callus culture. Euphytica 25: 393-403.

Linsmaier, E. M. and F. Skoog. 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol. Plant. 18: 100-127.

Love, R. M. 1938 Somatic variation of chromosome numbers in hybrid wheats. Genetics 23: 517-522.

Makino, S. 1957 The chromosome cytology of the ascites tumors of rats, with special reference to the stemline cell. Int. Rev. Cytol. 6: 25-84.

Matsubayashi, M. 1963 Prolonged direct staining of aceto-carmine squashes of Solanum microsporocytes. Stain Technol. 38: 264-266.

Melchers, G. 1974 Haploids for breeding by mutation and recombination. In "Polyploidy and Induced Mutations in Plant Breeding". I.A.E.A., Vienna

Melchers, G. and L. Bergmann. 1959 Untersuchungen an Kulturen von haploid Geweben von Antirrhinum majus. Ber. Deut. Bot. Ges. 71: 459-473.

Melchers, G. and G. Labib. 1974 Somatic hybridization of plants by fusion of protoplasts. I. Selection of light resistant hybrids of "haploid" light sensitive varieties of tobacco. Molec. Gen. Genet. 135: 277-294.

Miller, C. O., F. Skoog, M. H. Saltz and F. M. Strong. 1955 Kinetin, a cell division factor from desoxyribonucleic acid. J. Amer. Chem. Soc. 77: 1392.

Mitra, J., M. O. Mapes and F. C. Steward. 1960 Growth and organized development of cultured cells. LV. The behavior of the nucleus. Amer. J. Bot. 47: 357-368.

Moav, R. 1957 Somatic chromosome instability in Nicotiana hybrids. Ph. D. Dissertation, University of California.

Mok, D. W. S. and S. J. Peloquin. 1975a Three mechanisms of 2n pollen formation in diploid potatoes. Can. J. Genet. Cytol. 17: 217-225.

Mok, D. W. S. and S. J. Peloquin. 1975b The inheritance of three mechanisms of diplandroid (2n pollen) formation in diploid potatoes. Heredity 35: 295-302.

Morel, G. and G. Martin. 1952 Guerison de dahlias atteints d'une malade a virus. C. R. hebd. Seanc. Acad. Sci. Paris 235: 1324-1325.

Muir, W. H., A. C. Hildebrandt and A. J. Riker. 1954 Plant tissue cultures produced from single isolated cells. Science 119: 877-878.

Murashige, T. and R. Nakano. 1967 Chromosome complement as a determinant of the morphogenetic potential of tobacco cells. Amer. J. Bot. 54: 963-970.

Nagata, T. and I. Takebe. 1970 Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92: 301-308.

Nagata, T. and I. Takebe. 1971 Plating of isolated tobacco mesophyll protoplasts on agar medium. Ibid. 99: 12-20.

Nakata, K. and M. Tanaka. 1968 Differentiation of embryoids from developing germ cells in anther culture of tobacco. Japan. J. Genet. 43: 65-71.

Niizeki, H. and K. Oono. 1968 Induction of haploid rice plant from anther culture. Proc. Japan Acad. 44: 554-556.

Niizeki, M. 1974 Studies on plant cell and tissue culture. V. Effect of different kinds of media on the variation of chromosome number in tobacco callus and regenerated plant. J. Fac. Agr., Hokkaido Univ. (Sapporo) 57: 357-367.

Niizeki, M. and W. F. Grant. 1971 Callus, plantlet formation and polyploidy from cultured anthers of Lotus and Nicotiana. Can. J. Bot. 49: 2041-2051.

Niizeki, M. and F. Kita. 1975 Effect of different chromosome numbers of tobacco pollen on plant formation by anther culture and fertilization. Japan J. Breed. 25: 258-264.

Nishi, T., Y. Yamada and E. Takahashi. 1973 The role of auxin in differentiation of rice tissues cultured in vitro. Bot. Mag. Tokyo 86: 183-188.

Nishiyama, I. and T. Taira. 1966 The effects of kinetin and indoleacetic acid on callus growth and organ formation in two species of Nicotiana. Japan. J. Genet. 41: 357-365.

Nobecourt, P. 1939 Sur la perennite et l'augmentation de volume des cultures de tissus vegetaux. C. r. Seanc. Soc. Biol. 130: 1270-1271.

Norstog, K., W. E. Wall and G. P. Howland. 1969 Cytological characteristics of ten-year-old rye-grass endosperm tissue cultures. Bot. Gaz. 130: 83-86.

Novak, F. J. and B. Vyskot. 1975 Karyology of callus cultures derived from Nicotiana tabacum L. haploids and ploidy of regenerates. Z. Pflanzenzuetg. 75: 62-70.

Ogura, H. 1975a The effects of a morphactin, chlorflurenol, on organ redifferentiation from tobacco calluses cultured in vitro. Bot. Mag. Tokyo 88: 1-8.

Ogura, H. 1975b Morphactin-kinetin interaction on growth and shoot formation in tobacco callus cultures. Plant and Cell Physiol. 16: 563-569.

Ogura, H. 1975c The role of morphactin in cultured tissues. Naturwissenschaften 62: 393.

Ogura, H. 1976 The cytological chimeras in original regenerates from tobacco tissue cultures and in their offsprings. Japan. J. Genet. 161-174.

Ogura, H. 1977 Considerations on the involvements of genetical differences with callus formation in wheat. Wheat Inf. Serv. 44: 5-7.

Ogura, H. 1977 Genetic control of chromosomal chimerism found in a regenerate from tobacco callus. Japan. J. Genet., submitted.

Ogura, H. and K. Tsunewaki. 1974 Anther culture of the cytoplasm substitution lines of Triticum aestivum cv. Chinese Spring. Wheat Inf. Serv. 39: 13-14.

Ogura, H. and S. Tsuji. 1977 Differential responses of Nicotiana tabacum L. and its putative progenitors to de- and redifferentiation. Z. Pflanzenphysiol. 84: 195-202.

Ohyama, K., O. L. Gamborg and R. A. Miller. 1972 Uptake of DNA by plant protoplasts. Can. J. Bot. 50: 2077-2080.

Omara, M. K. 1976 Cytomixis in Lolium perenne. Chromosoma 55: 267-271.

Otsuki, Y. and I. Takebe. 1969 Isolation of intact mesophyll cells and their protoplasts from higher plants. Plant and Cell Physiol. 10: 197-201.

Pieniazek, J. and M. Saniewski. 1968 The synergistic effect of benzyladenine and morphactin on cambial activity in apple shoots. Bull. Acad. Pol. Sci. 16: 381-384.

Power, J. B., S. E. Cummins and E. C. Cocking. 1970 Fusion of isolated protoplasts. Nature 225: 1016-1018.

Power, J. B., E. M. Frearson, C. Hayward, D. George, P. K. Evans, S. F. Berry and E. C. Cocking. 1976 Somatic hybridization of Petunia hybrida and P. parodii. Nature 263: 500-502.

Robbins, W. J. 1922 Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz. 73: 376-390.

Robbins, W. J. and M. A. Bartley. 1937 Vitamin B_1 and the growth of excised tomato roots. Science 85: 246-247.

Ruesink, A. W. and K. V. Thimann. 1965 Protoplasts from the Avena coleoptile. Proc. Natl. Acad. Sci. U.S.A. 54: 56-64.

Ruesink, A. W. and K. V. Thimann. 1966 Protoplasts: preparation from higher plants. Science 154: 280-281.

Sacristan, M. D. and G. Melchers. 1969 The caryological analysis of plants regenerated from tumorous and other callus cultures of tobacco. Molec. Gen. Genet. 105: 317-333.

Sakai, K. and Y. Shimamoto. 1975 Developmental instability in leaves and flowers of Nicotiana tabacum. Genetics 51: 801-813.

Sankhla, D. and N. Sankhla. 1968 Morphactin-gibberellin interaction in lettuce seed germination and seedling growth. Biol. Plant. (Praha) 10: 37-40.

Schneider, G. 1964 Eine neue Gruppe von synthetischen Pflanzenwachstumsregulatoren. Naturwissenschaften 51: 416-417.

Schneider, G. 1967 Wirkung von Morphaktinen auf Kallus- und adventivwurzelbildung an Hypokotylzylindern von Phaseolus vulgaris L. Wiss. Z. Univ. Rostock, Math.-Naturwiss. 16: 699-702.

Schneider, G. 1970 Morphactins: physiology and performance. Ann. Rev. Plant Physiol. 21: 499-536.

Schott, H. H. and H. Schraudolf. 1967 Die Wirkung einiger Derivate der 9-Flurenol-9-Carbonsaure auf die Regeneration von Begoniablattscheiben (Begonia rex). Z. Pflanzenphysiol. 56: 387-396.

Sheen, S. J. 1972 Isozymic evidence bearing on the origin of Nicotiana tabacum L. Evolution 26: 143-154

Shimada, T. and M. Tabata. 1967 Chromosome numbers in cultured pith tissue of tobacco. Japan. J. Genet. 42: 195-201.

Shimada, T. and T. Makino. 1975 In vitro culture of wheat. III. Anther culture of the A genome aneuploids in common wheat. Theor. Appl. Genet. 46: 407-410.

Singh, B. D., B. L. Harcey, K. N. Kao and R. A. Miller. 1972 Selective pressure in cell populations of Vicia hajastana cultured in vitro. Can. J. Genet. Cytol. 14: 65-70.

Skoog, F. 1954 Substances involved in normal growth and differentiation of plants. Brookhaven Symp. Biol. 6: 1-21.

Skoog, F. 1970 Aspects of growth factor interactions in morphogenesis of tobacco tissue cultures. In "Les Culture de Tissus de Plantes." pp. 115-136. Colloques Internationaux du C.N.R.S. no. 193, Paris.

Skoog, F. and C. Tsui. 1948 Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exptl. Biol. 11: 118-140.

Smith, H. H., K. N. Kao and N. C. Combatti. 1976 Interspecific hybridization by protoplast fusion in Nicotiana: Confirmatio and development. J. Hered. 67: 123-128.

Speckman, G. J. and G. E. Dijk. 1972 Chromosome number and plant morphology in some ecotypes of Poa pratensis L. Euphytica 21: 171-180.

Steward, F. C. 1958 Growth and organized developmetn of cultured cells. III. Interprtations of the growth from free cell to carrot plant. Amer. J. Bot. 45: 709-713.

Steward, F. C., M. O Mapes and J. Smith. 1958 Ditto. I. Growth and division of freely suspended cells. Ibid. 45: 693-703.

Steward, F. C., M. O. Mapes and K. Mears. 1958 Ditto II. Organization in cultures grown from freely suspended cells. Ibid. 45: 705-708.

Straus, J. 1954 Maize endosperm tissue grown in vitro. II. Morphology and cytology. Amer. J. Bot. 41: 833-839.

Street, H. E. 1973 Old problems and new perspectives. In "Plant Tissue and Cell Culture" Ed. H. E. Street., Blackwell Scientific Publications, Oxford.

Tabata, M. and F. Motoyoshi. 1963 Hereditary control of callus formation in maize endosperm cultured in vitro. Japan. J. Genet. 40: 343-355.

Tabata, M., H. Yamamoto and N. Hiraoka. 1968 Chromosome constitution of mature plants derived from cultured pith of tobacco. Japan. J. Genet. 43: 319-322.

Takebe, I., Y. Otsuki and S. Aoki. 1968 Isolation of tobacco mesophyll cells in intact and active state. Plant and Cell Physiol. 9: 115-124.

Takebe, I., Y. Otsuki and S. Aoki. 1971 Infection of isolated mesophylll protoplasts by tobacco mosaic virus. In "Les Culture de Tissus de Plantes" pp. 503-511. Colloque Internationaux du C.N.R.S., No. 193. Paris.

Tanaka, M. and K. Nakata. 1969 Tobacco plants obtained by anther culture and the experiment to get diploid seeds from haploids. Japan. J. Genet. 44: 47-54.

Taylor, J. H. 1950 The duration of differentiation in excised anthers. Amer. J. Bot. 37: 137-143.

Torrey, J. G. 1957 Cell division in isolated single cells in vitro. Proc. Natl. Acad. Sci. U.S.A. 43: 887-891.

Torrey, J. G. 1959 Experimental modification of development in the root. In "Cell, Organism and Milieu" pp. 189-222. Ed. D. Rudnick, Ronald Press, New York.

Torrey, J. G. 1967 Morphogenesis in relation to chromosomal constitution in long-term plant tissue cultures. Physiol. Plant. 20: 265-275.

Tulecke, W. 1957 The pollen of Ginkgo biloba: In vitro culture and tissue formation. Amer. J. Bot. 44: 602-608.

Tulecke, W. 1959 The pollen cultures of C. D. La Rue: A tissue from the pollen of Taxus. Bull. Torrey Bot. Club 86: 283-289.

Vaarama, A. 1949 Spindle abnormalities and variation in chromosome number in Ribes nigrum. Hereditas 35: 136-162.

Van Overbeek, J., M. E. Coklin and A. F. Blakeslee. 1941 Factors in coconut milk essential for growth and development of very young Datura embryos. Science 94: 350-351.

Vasil, V. and A. C. Hildebrandt. 1967 Further studies on the growth and differentiation of single, isolated cells of tobacco in vitro. Planta 75: 139-151.

Venketeswaran, S. and E. B. Spiess. 1964 Tissue culture studies on Vicia faba. IV. Effect of growth factors on mitotic activity. Cytologia 29: 298-310.

Wain, R. L. 1958 Relation of chemical structure to activity for 2,4-D type herbicide and plant growth regulators. In "Advances in Pest Control Research 2" pp. 263-305, Ed. R. L. Metcalf, Interscience Publishers Inc., New York, N. Y.

Watanabe, Y. 1962 Chromosome-mosaics observed in a variety of wheat "Shirahada." Japan. J. Genet. 37: 194-206.

Went, F. W. and K. V. Thimann. 1937 Phytohormones. Macmillan Co., New York.

White, P. R. 1934 Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. (Lancaster) 9: 585-600.

White, P. R. 1939a Potentially unlimited growth of excised plant callus in an artificial medium. Amer. J. Bot. 26: 59-64.

White, P. R. 1939b Controlled differentiation in a plant tissue culture. Bull. Torrey Bot. Club 66: 507-513.

White, P. R. 1943 Nutrient deficiency studies and an improved inorganic nutrient for cultivation of excised tomato roots. Growth 7: 53-65.

White, P. R. 1949 Prolonged survival of excised animal tissues in vitro in nutrients of known composition. J. Cell. Comp. Physiol. 34: 221-242.

White, P. R. and A. C. Braun. 1941 Crown-gall production by bacteria-free tumor tissues. Science 94: 239-241.

Yamane, Y. 1975 Chromosomal variation in calluses induced in Vicia faba and Allium cepa. Japan. J. Genet. 50: 353-355.

Yang, S. J. 1964 Numerical chromosome instability in Nicotiana hybrid. I. Interplant variation among offspring of amphidiploid. Genetics 50: 745-756.

Yang, S. J. 1965 Ditto. II. Intraplant variation. Can. J. Genet. Cytol. 7: 112-119.

Zagorska, N. A., Z. B. Shamina and R. G. Butenko. 1974 The relationship of morphogenetic potency of tobacco tissue culture and its cytogenetic features. Biol. Plant. (Praha) 16: 262-274.

Ziegler, H. 1970 Morphactins. Endeavour 29: 112-116.